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(71) Applicant (for all designated States except US): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): SETH, Prem, K. [IN/US]; 11705 Cherry Grove Drive, North Potomac, MD 20878 (US). COWAN, Kenneth [US/US]; 12005 Gregersaroft Road, Potomac, MD 20854 (US).			Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: METHODS OF PREPARATION AND USE OF RECOMBINANT ADENOVIRAL VECTORS			
(57) Abstract <p>The present invention describes novel methods of constructing recombinant adenoviral vectors capable of expressing human cDNAs, such as wild-type p53, WAF1/Cip1/p21, p27/kip1, <i>E. coli</i> cytosine deaminase, wild-type p16, TAM 67 (a jun/fos dominant negative mutant) and B7-1 and B7-2. The invention further provides methods of inhibiting the proliferation of cells, inhibiting the cell cycle of proliferating cells, and methods for the eradication of cells, especially cancer and diseased cells, by infecting the cells with a recombinant adenovirus vector capable of expressing human cDNAs. Compositions and methods of the invention are suitable for treatment of a subject afflicted with a tumor wherein the cells of the tumor, for example, lack the wild-type p53 allele and/or possess a mutated p53 gene. The invention additionally provides a method for the use of adenoviral vectors in the treatment of cancer cells, such as lung cancer and breast cancer cells. The invention further provides methods for the use of adenoviral vectors in cancer gene therapy as a mechanism for purging bone marrow cells of contaminating tumor cells, for eradicating cancer cells, and for preventing development of cancer cells and tumors.</p>			

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**METHODS OF PREPARATION AND USE OF
RECOMBINANT ADENOVIRAL VECTORS**

FIELD OF THE INVENTION:

5 The invention describes novel methods of constructing
recombinant adenoviral vectors capable of expressing human
cDNAs, and methods of use for inhibiting proliferation of
cells and methods of eradicating cells. Compositions and
methods of the invention are suitable for treatment of a
subject afflicted with a tumor and are also useful in cancer
10 gene therapy as a mechanism for purging bone marrow cells of
contaminating tumor cells, and preventing the development of
cancer cells and tumors.

BACKGROUND OF THE INVENTION:

15 In recent years, recombinant adenoviruses have become
a popular tool for the study of both adenoviral biology and
in vitro and in vivo gene transfer. For gene therapy
purposes, adenoviral vectors have been rendered replication-
deficient by replacing the replication regulating Ela
20 nucleotide sequence located at the 5' end of the adenovirus
genome with foreign gene expression cassettes.

To replace the portion of the Ela nucleotide sequence
in the adenoviral genome with the foreign gene expression
cassette, one must modify the adenoviral genome so that
25 adenovirus virions are not produced. These modifications
are known in the art, and have been achieved through the
application of two methods. One method utilizes the
presence of a unique ClaI restriction site to excise 900
base pairs of the 5' end of the adenovirus genome (2.6 map
30 units of the adenovirus genome). The remaining nucleotide
sequence of the adenovirus genome, devoid of its 5' end,
does not produce virions as it has lost two essential
elements critical for the replication of adenovirus genome:
(1) the left inverted terminal repeat; and (2) half of the

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0 Ela sequences. Perricaudet and their colleagues pioneered
this single ClaI digestion technique (mainly using genomic
DNA of d1327 mutant of Ad5) and succeeded in constructing
adenoviral vectors. (See Stratford-Perricaudet, L. D.,
5 Makeh, I., Perricaudet, M. and Briand, P. J., J. Clin.
Invest. 90:626-630 (1992)).

10 However, the single ClaI digestion technique poses
several problems. Initially, there are difficulties in the
isolation of recombinant adenovirus due to the high
background of parental genome (d1327). When d1327 DNA is
cut with ClaI restriction enzyme, uncut DNA still remains.
Because the difference between the genomic DNA sizes of the
desired (32 kb) and undesired (33 kb) DNA is only about 1
kb, it is virtually impossible to separate the two DNA
15 fragments derived from the partial digestion of the
adenovirus genome. To circumvent this problem, one must
screen a large number of possible recombinant adenoviruses
to rescue a single recombinant adenovirus.

20 Another approach for modification of the adenovirus
genome is by the replacement of DNA sequences in the
adenoviral genome by another DNA fragment, resulting in a
adenoviral genomic sequence large enough to exceed the
packaging limit of the adenovirus virions. This problem has
been addressed through a method which replaces the 2.2 kb
25 fragment of pFG140 (a circular DNA derived from d1309 genome
with 4.4-kb DNA fragment) containing an ampicillin
resistance gene and a bacterial origin of replication (See
Graham, F. L. and Prevec, L. (1991) Manipulation of
adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene
30 transfer and expression protocols, Humana Press, Clifton,
New Jersey)). The resulting plasmid, designated pJM17, may
then be propagated as a plasmid. pJM17 can be rescued as
infectious virions when the foreign 4.4 kb fragment of pJM17
is replaced by homologous recombination with another DNA
35 fragment small enough for the resulting genome to package
the adenovirus virion.

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Although the pJM17 system solves the background problem present during the screening of recombinant adenoviruses, the difficulty in the quality control of the plasmids and the recombinant vectors still remains. This may be due to the large size and structure of pJM17. pJM17 is a very large circular DNA molecule (about 40 kb) and has a tendency to undergo undefined rearrangements. After such homologous recombination, specific DNA sequences are occasionally kicked out from the recombinant constructs in vivo, thus rendering the expression of the DNA inserts impossible.

The present invention provides a novel method for the construction of adenoviral vectors. The invention provides a technique of constructing an adenoviral vector whereby an additional ClaI restriction site is introduced upstream of the original ClaI site. The introduction of the second ClaI restriction site greatly reduces the chances of obtaining undigested DNA genome. Because only one of the two ClaI sites must be cut to prevent the production of the non-recombinant background infectious virions, the addition of a second ClaI site greatly increases the chances of generating fully cut DNA and thus reducing the parental genome background. Moreover, because the adenoviral vectors are made using viral genomic DNA as the starting point, there is no need to utilize a plasmid based vector.

Adenoviral vectors are generally the preferred vector for the expression of DNA fragments. Although plasmids and retroviruses have been used to express DNA fragments, the efficiency of transfection is generally low. (See Chen, et al., Science, 250:1576-1579, (1990); Shaw, et al., Proc. Natl. Acad. Sci. USA, 89:4495-4499, (1992); and Casey, et al., Oncogene, 6:1807-1811, (1991)). Adenoviral vectors are the preferred vector because they possess certain characteristics which allow for a high efficiency of transfection. Adenovirus based vectors are capable of a high efficiency of transfections because 1) they can grow to high titers; 2) they are internalized into cells with an

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efficient receptor-mediated endocytosis; 3) are replication incompetent; and 4) they express a transgene to high levels in epithelial cells. (Ginsberg, H. S., *Virology*, eds. Dulbecco, R. and Ginsberg, H. S., (Lippincott, Co., Philadelphia) pp 147-160 (1988); Graham, et al., In *Gene transfer and expression protocols*, (Murray, E. J. ed.) pp. 109-128, Humana Press, Clifton, New Jersey (1991); Seth, et al., *Virus attachment and entry into cells*, eds. Colwell, R. L., and Lonberg-Holm, K. (American Society for Microbiology, Washington, D.C.) pp 191-195, (1986); Seth, et al., *J. Virol.* 68:933-940 (1994); Rosenfeld, et al., *Hum. Gene Ther.* 5:331-342 (1994)).

Methods of constructing adenoviral vectors capable of expressing of cDNA fragments, such as wild-type p53, WAF1/Cip1/p21, wild-type p16, p27/kip1, and *E. coli* cytosine deaminase are provided by the present invention. The utilization of adenoviral expression vectors capable of producing high levels of proteins in cells allows for the study of the roles of these proteins in the control and regulation of cell growth in both normal and malignant cells. Furthermore, this strategy has implications in gene therapy for cancers.

It is commonly known that the protein encoded by the wild-type p53 gene affects cell proliferation by recognizing DNA damage to a cell, resulting in either a delay in progress through the cell cycle to allow for the repair processes of the cell to proceed, or by the initiation of programmed cell death, and/or the induction of apoptosis. See Kuerbitz, et al., *Proc. Natl. Acad. Sci. USA*, 89:7491-7495 (1992); Kastan, et al., *Cancer Res.*, 51:6304-6311, (1991); Lowe, et al., *Cell*, 74:957-968 (1993); and Levine, et al., *Br. J. Can.*, 69:409-416 (1994).

For those cells which express mutant p53, the effects of wild-type p53 are abrogated, resulting in abnormal cell growth and an increase the number of cells leading to cancer. Those cancers which have a high percentage of p53

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mutations and selection against the wild-type p53 protein are, among others, those arising in the colon, lung, bladder, prostate, breast, and skin. See Hollstein, et al., Science, 253:49-53, (1991); and Levine, et al., Nature, 253:453-456, (1991). Recently, there has been an increasing interest in elucidating the mechanisms by which p53 mediates its functions in normal cells, how various mutations in p53 are responsible for aberrant cell growth, and in the possibility of employing wild type p53 in gene therapy. See Nigro, et al., Nature (Lond.), 342:705-708 (1989); Chen, et al., Science, 250:1576-1579, (1990); and Gottesman, M.M., J. Natl. Cancer Inst., 86:1277-1285, (1994). It is therefore important to understand the biological consequences of overexpression of the wild-type p53 gene in both normal and tumor cells.

A number of approaches have been employed in the study of the effects of p53 expression in cells, including the exposure of cells to DNA-damaging agents such as ultraviolet radiation and chemicals that react with DNA, both of which have been shown to induce increased expression of cellular p53. See Clarke, et al., Nature, 362:849-852 (1993); Lowe, et al., Nature (Lond.), 362:847-849, (1993); and Dulic, et al., Cell, 76:1013-1023, (1994).

Genetic approaches have similarly been used to study the effects of p53 expression in cells. These approaches specifically include the introduction of temperature sensitive mutants of p53 or gene knock-out experiments to alter intracellular p53 expression and function. See Michalovitz, et al., Cell, 62:671-680, (1990); Chiou, et al., Mol. Cell. Biol., 14:2556-2563, (1994); and Donehower, et al., Nature 356:215-221, (1992).

It has been shown that p53 expression can transcriptionally activate several genes, including WAF1/Cip1/p21, which was also independently isolated as a negative growth regulatory gene. (Xlong, et al., Nature 366: 701-704, (1993); El-Deiry, et al., Cell 75:817-825, (1993);

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Harper, et al., Cell 75:805-816, (1993)). Specifically, when DNA damage is induced, the intracellular levels of p53 rise and induce the expression of WAF1/Cip1. p21, the protein encoded by WAF1/Cip1, then binds to cyclin-dependent kinases and inhibits their activity. This event enables the cell cycle to be arrested before DNA synthesis, giving the cell the opportunity to repair the damaged DNA.

Thus, the utilization of an expression vector capable of producing high levels of WAF1/Cip1/p21 protein in cells would be useful in the determination of the exact relationship between p53 and WAF1-mediated cell growth regulation causing cell cycle arrest and/or apoptosis. Further, utilization of this vector would be useful in the determination of the effects of WAF1/Cip1/p21 gene expression in the absence of other p53-mediated signal transducing agents.

An additional area in which tools are needed for examination of the effects of proteins on cancer is in the area of vascular diseases. Vascular smooth muscle cells constitute the medial layer of arterial walls that maintains the normal tonus and resistance of vessels. The abnormality of vascular smooth muscle cells induces both functional and anatomical changes of the vessels (Ross, R. (1986) New Engl. J. Med. 314:488-500; Isoyama, S., et al., (1989) J. Clin. Invest. 84:288-294; and Ogata, M., et al. (1992) Am. J. Physiol. 262:H691-697), and these abnormalities could, in turn, give rise to a variety of serious stresses to the heart (Katayose, D., et al. (1993) Biochem. Biophys. Res. Commun. 191:587-594; Sandoval, J., et al., (1994) Circulation 89:1733-1744; and Tajima, M., et al., (1994) Cardiovasc. Res. 28:312-319). In anatomical changes of the vessel walls in vascular disease such as arteriosclerosis, pulmonary hypertension and vascular injury after angioplasty (Ross, R. (1986) New Engl. J. Med. 314:488-500; Botney, M.D., et al. (1994) Am. J. Pathol. 144:286-295; Katayose, D., et al., (1993) Am. J. Physiol. 264:L100-L106; and Speir,

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0 E., et al., (1994) Science 265:391-394), increased
cellularity by vascular smooth muscle cell proliferation are
observed. Given this knowledge, one of the possible
strategies for controlling vascular diseases is to decrease
the cellularity of vessel walls by letting vascular smooth
5 muscle cells undergo cell cycle arrest and/or apoptosis.

Adenoviral vector constructs that would be useful in
the understanding of the biochemical mechanisms underlying
the cell cycle progression through various stages are those
that express cyclin kinase inhibitors. A key contribution
10 has been the cloning of several cyclin kinase inhibitors,
such as p21/WAF1/Cip1, p27/kip1, and p16/INK4. Recent work
has indicated that each one of these kinase inhibitors can
potentially regulate one or more of the cyclin kinases
leading to the dephosphorylation of Rb protein, which in
15 turn can control the progression of the cell cycle into S
phase. While other RB-like proteins such as p107 and p130
can also potentially control this signal transduction
pathway leading to the cell cycle arrest, it has been
suggested that, at least for p16/INK4, mediated growth
20 arrest is tightly associated with the status of Rb protein
phosphorylation. Therefore, it would be extremely useful to
have adenoviral vectors that express p16/INK4 in order to
investigate the association between Rb and p16-mediated cell
cycle arrest. An additional adenoviral vector construct
25 that would be useful in the understanding of the biochemical
mechanisms that control growth regulation is an adenoviral
vector that expresses the cyclin kinase p27/kip1.

Adenoviral vector constructs that would be useful in
exploring the clinical utility of suicidal enzymes for the
30 gene therapy of breast cancer is one which expresses E. coli
cytosine deaminase. While adenoviral vectors have many
attractive features, a key problem with adenoviral vectors
is that they can only infect a small population of cancer
cells within a tumor mass, leaving many of the cells
35 uninfected. Thus, there is a need to develop adenoviral

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vectors which should induce cytotoxicity across the whole
tumor should adenovirus infect only a small number of tumor
cells. One approach would be to use adenoviral vectors
which under certain circumstances can be made to produce
cytotoxic products which are smaller in size and hence will
5 have opportunities to escape the cells and kill the
uninfected cells.

There are other applications in which adenoviral
vectors would prove to be extremely useful. For example,
during acute chemotherapy, many breast cancer patients often
10 acquire resistance to various drugs. Although a great deal
is known about the molecular mechanisms by which tumor cells
acquire drug resistance, there are relatively limited
approaches to treat cancers once the drug resistance has
been acquired. Thus, there is a need for approaches for
15 treating drug resistant cancers.

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SUMMARY OF THE INVENTION:

The invention provides for methods of constructing recombinant adenoviral vectors capable of expressing human cDNAs, such as wild-type p53 cDNA, called herein AdWTP53; WAF1/Cip1 cDNA, called herein AdWAF1; p27/kip1, called
5 herein Adp27; E. coli cytosine deaminase, called herein AdCD; wild-type p16, called herein Adp16; TAM 67 (a jun/fos dominant negative mutant), called herein AdTAM67; and B7-1 and B7-2, called herein AdB7-1 and AdB7-2, respectively.

10 The invention further provides a method of inhibiting the growth and/or the cell cycle of proliferating cells. This method comprises contacting the cells with a recombinant adenovirus vector capable of expressing human cDNAs in an amount effective to inhibit cell proliferation.

15 The invention also provides a composition for contacting cells with an amount of a recombinant adenovirus vector capable of expressing human cDNAs in an amount effective to inhibit cell proliferation.

20 The invention additionally provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of a recombinant adenovirus vector capable of expressing human cDNAs so as to inhibit proliferation of the tumor cells.

25 Further, the invention provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of a recombinant adenovirus capable of expressing human cDNAs in the presence of a chemotherapeutic agent so as to inhibit proliferation of the tumor cells.

30 Additionally, the invention provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of a recombinant adenovirus capable of expressing human cDNAs in the presence of an amount of irradiation so as to inhibit proliferation of the tumor cells.

35 The invention also provides for the use of adenoviral

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° vectors in cancer gene therapy as a mechanism for purging bone marrow cells of possible breast cancer contaminants.

5 The invention also provides for the use of adenoviral vectors in combination with toxins and cytotoxic drugs as a mechanism for purging bone marrow cells of possible breast cancer contaminants.

10 The invention further provides for the use of adenoviral vectors to eradicate cancer cells and tumors by contacting the cancer cells with an amount of adenoviral vector sufficient to eradicate the cancer cells.

Further, the invention provides for the use of adenoviral vectors as a preventative mechanism for the development of cancer in subjects who are at risk of developing cancer.

15 The invention also provides for a method of treating a subject afflicted with a tumor that has shown resistance to drugs which comprises contacting the tumor with an effective amount of a recombinant adenovirus capable of expressing human cDNAs so as to inhibit proliferation of the tumor cells.

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BRIEF DESCRIPTION OF THE FIGURES:

How these and other objects of this invention are achieved will become apparent in the light of the accompanying disclosure and with reference to the accompanying drawings.

Figure 1: Structure of the Recombinant Adenoviral Vector AdWtp53. A diagrammatical representation of the structure of the recombinant adenoviral vector AdWtp53 is shown. The top, hatched segment of the diagram represents the adenovirus type 5 genome, consisting approximately of 9.24-100 map units (mu). The bottom portion of the diagram represents an enlargement of the human-wild type p53 expression cassette. The human-wild type p53 expression cassette contains a left inverted terminal repeat (ITR), an origin of replication, encapsidation signals, and an Ela enhancer derived from adenovirus type 5 (stippled segments). The expression cassette also contains human wild-type p53 cDNA (solid segments) and an SV40 maturation signal (right blank segment).

Figure 2A: Immunoprecipitation of ³⁵S-labeled human wild-type p53 protein from H-358 cells exposed to varying doses of AdWtp53 or AdControl. This figure shows the immunoprecipitation of ³⁵S-labeled human wild-type p53 protein from H-358 cells exposed to increasing doses of AdWtp53 or AdControl. After labeling the cells with ³⁵S-methionine-cysteine, cell lysates were immunoprecipitated using anti-p53 antibody, solubilized protein samples loaded on 8% SDS-polyacrylamide gel electrophoresis, gels dried and exposed to X-ray film. The left panel shows radioactive signals of p53 precipitates from H-358 cells exposed to AdControl at 0.1, 1, 10 and 50 pfu/cell. The numbers 0.1 through 50 denoted above the lanes represent the pfu/cell. The arrow indicates the position of migration of p53 protein.

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Figur 2B: Immunoprecipitation of ³⁵S-labeled p53 protein from various cell lines. Cell lines, MCF-10, MCF-7, MDA-MB-231 and Adr^R MCF-7 were exposed to AdWTp53 (50 pfu/cell) or AdControl (adenovirus alone; 50 pfu/cell), and p53 protein was immunoprecipitated as set forth in Example 8. The left panel shows the results of p53 immunoprecipitation of uninfected cells, the middle panel shows the immunoprecipitation of cell exposed to AdControl, and the right panel indicates the results of immunoprecipitation of cells exposed to AdWTp53. The arrow indicates the position of migration of p53 protein.

Figure 3A: Effect of AdWTp53 and AdControl on H-358 cell growth. 5×10^4 cells were plated in triplicate on 6 well tissue culture plates, exposed to AdWTp53 (10 pfu/cells) or AdControl (10 pfu/cell), and the cell number counted on each day. Shown are cell number of H-358 cells: uninfected (), exposed to AdWTp53 (■), and exposed to AdControl (■). Values shown are mean \pm SE.

Figure 3B: Effect of AdWTp53 and AdControl on MDA-MB-231 cell growth. 5×10^4 cells were plated in triplicate on 6 well tissue culture plates, exposed to AdWTp53 (10 pfu/cells) or AdControl (10 pfu/cell), and the cell number counted on each day. Shown are cell number of MDA-MB-231 cells: uninfected (●), exposed to AdWTp53 (■), and exposed to AdControl (■). Values shown are mean \pm SE.

Figure 3C: Effect of AdWTp53 and AdControl on MCF-7 cell growth. 5×10^4 cells were plated in triplicate on 6 well tissue culture plates, exposed to AdWTp53 (10 pfu/cells) or AdControl (10 pfu/cell), and the cell number counted on each day. Shown are cell number of MCF-7 cells: uninfected (), exposed to AdWTp53 (■) exposed to AdControl (■). Values

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shown are mean \pm SE.

Figure 4: Effect of AdWtp53 on the survival rate of different cells. Cells were exposed to different concentrations of AdWtp53 for 7 days and the survival rates calculated by the colorimetric method described in Example 3. Shown are the percent survival values for each cell line using different pfu/cell, as shown in Figure 3. The results for each cell line are represented by the following symbols: MDA-MB-157 (■), H-358 (◆), MDA-MB-231 (●), MDA-MB-453 (▲), MCF-7 (●), MCF-10 (■), 184B5 (▲), NMECs (◆), and represent the mean of triplicate determinations.

Figure 5: Western blot analysis of p53, WAF1/Cip1, mdm2 and actin proteins in breast cancer cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-453, MCF-7), a lung cancer cell line (H-358), immortalized mammary cells (MCF10, 184B5) and NMECs. Cells at a concentration of 0.5×10^6 were plated in 6 cm tissue culture dishes and infected with either 10 or 50 pfu/cell of AdWtp53 or 50 pfu/cell of AdControl for 24 hours. The cells were harvested and resuspended in 1 ml of 1xSDS-polyacrylamide gel electrophoresis buffer and 15 μ g of protein were separated in a 8% SDS polyacrylamide gel, electroblotted onto nitrocellulose, and the membranes reacted with antibodies corresponding to p53, WAF1/Cip1, mdm2 and actin. Protein bands were detected by autoradiography of X-ray film. The type of each cell line used is shown on the top of the panel. Numbers 10 or 50 on top of the lanes represent the amount of AdControl or AdWtp53 (pfu/cell). The antibodies used for detecting proteins are indicated on the left side of the panel. Protein molecular weight markers are indicated on the right side of the panel.

Figure 6: Northern blot analysis of p53 mRNA in cells

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exposed to AdWtp53. Cells (2×10^6) were plated and 24 hours later infected with AdWtp53 or AdControl (10 pfu/cell). 24 hours after infection, RNA was prepared and subjected to Northern blot analysis as described in Example 6. After transferring RNA to Magna NT membranes, blots were either probed with a p53 or a 36B4 cDNA probe. The results of autoradiograms obtained from different cells are shown on top of the lane, exposed to either AdControl or AdWtp53 as shown.

Figure 7: Nucleosomal DNA fragmentation in AdWtp53-infected MDA-MB-231, MCF-7 and NMECs. 2×10^6 cells were plated in 10 cm dishes and exposed to either AdControl or AdWtp53. 1 day after infection, the cells were collected, incubated with a lysis buffer. Low molecular weight DNA was then prepared and subjected to an agarose gel electrophoresis. The results shown are the DNA pattern observed in various cell lines (shown on top of the lane) infected with 50 pfu/cell of either AdControl or AdWtp53. The numbers on the left side of the panel indicate the position of molecular weight markers (bp).

Figure 8: Construction of the recombinant adenoviral vector AdWAF1. A diagrammatical representation of the method of construction of the adenoviral vectors AdWtp53 and AdWAF1 is shown. The ClaI restriction site which was added by the invention is the upstream site, located within the first 900 nucleotides from the 5' end of the adenovirus genome.

Figure 9: Structure of the recombinant adenoviral vectors AdWtp53 and AdWAF1. This Figure represents the structure of the recombinant adenoviral vectors AdWtp53 and AdWAF1. On the top, the hatched segment represents adenovirus type 5 genome of 9.24 mu-100 mu. On the bottom is shown the enlargement of human wild type p53 and WAF1/Cip1 expression cassette. The expression cassettes contains left inverted

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terminal repeat (ITR), origin of replication, encapsidation signals and Ela enhancer derived from adenovirus type 5 (stippled segments); human cytomegalovirus immediate early promotor (left blank segment); human wild type p53 or WAF1/Cip1 cDNA (solid segment) and SV40 RNA maturation signal (right blank segment). AdWAF1 has the identical genomic structure as AdWTp53 except p53 cDNA is replaced by WAF1/Cip1 cDNA.

Figure 10: Western blot analysis of p53, WAF1/Cip1 and actin proteins in various cell lines following AdWTp53 and AdWAF1 infection. This Figure sets forth a Western blot analysis of p53, WAF1/Cip1 and actin proteins in various cell lines following AdWTp53 and AdWAF1 infection. The cells were infected with 50 pfu/cell of AdControl, AdWTp53 or AdWAF1 for 48 hours and subjected to Western blot analysis. The cell lines used are indicated on the top of the panel and the antibodies on the left side of the panel. The protein molecular weight markers are indicated on the right side of the panel.

Figures 11A-C: Effect of AdWTp53 and AdControl on cell growth. These Figures set forth the effect of AdWTp53 and AdControl on cell growth. The cells were exposed to AdWTp53 (10 pfu/cell), AdWAF1 (10 pfu/cell), and AdControl (10 pfu/cell), and the cell number was counted on each day. Shown are the cell number of H-358 cells (Figure 11A), MDA-MB-231 cells (Figure 11B) and MCF-7 cells (Figure 11C). The cell number in AdWTp53 infected cells are shown by (■), AdWAF1 infected cells by (●), AdControl infected cells by (■) and uninfected cells by (●). The values shown are mean \pm SE.

Figure 12A-D: Cell cycle analysis of MDA-MB-231 cells infected with AdWTp53 and AdWAF1. These Figures set forth

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a cell cycle analysis of MDA-MB-231 cells infected with AdWtp53 and AdWAF1. MDA-MB-231 cells were infected with AdControl, AdWtp53 or AdWAF1 (50 pfu/cell) and 24 hours later were subjected to cell cycle analysis as described in Example 13. The results shown are the cell cycle analysis of uninfected cells (Figure 12A); AdControl infected cells (Figure 12B); AdWtp53 infected cells (Figure 12C); and AdWAF1 infected cells (Figure 12D).

Figures 13A-D: Percentage distribution of cells in different cell cycle stages following AdWtp53 and AdWAF1 infection. These Figures set forth the percentage distribution of cells in different cell cycle stages following AdWtp53 and AdWAF1 infection. The cells were exposed to AdWtp53, AdWAF1 or AdControl and subjected to cell cycle analysis as described in Example 16. Shown are the percentage of cells in G1 (solid bars), S (hatched bars) and G2 + M (dashed bars) in MDA-MB-231 cells (Figure 13A); H-358 cells (Figure 13B); MCF-7 cells (Figure 13C); and NMECs (Figure 13D).

Figures 14A-E: Detection of Apoptosis in AdWtp53 and AdWAF1-infected Cells. These Figures set forth the detection of apoptosis in AdWtp53 and AdWAF1-infected cells. Two days after infection, adherent and floating cells were collected and incubated with a lysis buffer. Low molecular weight DNA was then prepared and analyzed by agarose gel electrophoresis. The results shown (Figures 14A-C) are the DNA pattern observed in various cell lines (as indicated on top of the lane) infected with 50 pfu/cell of either AdControl, AdWtp53 or AdWAF1. The positions of the molecular weight markers (bp) are indicated on the left side of the panel. In parallel experiments, MDA-MB-231 cells were also subjected to cell cycle analysis. Shown are the results following infection with AdWtp53 (Figure 14D) or AdWAF1 (Figure 14E) infection (10 pfu/cell, 24 hour

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infection). The arrow in Figure 14D indicates the position of apoptotic fragmented cells.

Figure 15: Ad.RSV β gal expression in MDA-MB-231 and human bone marrow cells. This Figure sets forth Ad.RSV β gal expression in MDA-MB-231 and human bone marrow cells. The cells (2×10^4) were infected with different moi of Ad.RSV β gal (0-10,000 pfu/cell) and β -gal activity determined as described in Example 7. The results show the β -gal activity obtained in MDA-MB-231 (●) and human bone marrow cells (o). The results indicate the average of the triplicate determinations.

Figure 16: Effect of AdWTp53 on the MDA-MB-231 and human bone marrow cell's viability. This Figure indicates the effect of AdWTp53 on the MDA-MB-231 and human bone marrow cell's viability. Freshly trypsinized MDA-MB-231 cells were mixed with CD34⁺ bone marrow cells and infected with different pfu/cell of AdWTp53. The cells were then assayed for colonogenicity as described in Example 19. The results shown are the percentage number of colonies formed after each treatment, assuming uninfected cells to be 100 %. The results of MDA-MB-231 cells are shown by (●) and of CD34⁺ cells by (o) and are the average of the triplicate determinations.

Figure 17: Ad-mediated transfection of CMV β -gal plasmid in MDA-MB-231 and human bone marrow cells. Cells (2×10^5) were transfected using CMV β -gal plasmid and different moi of dl312. After a 24 hour incubation at 37° C, the cells were lysed and β -gal activity measured. β -gal activity obtained in MDA-MB-231 cells is shown by (●) and in bone marrow cells by (o). The results are of the mean of the triplicate determinations.

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Figure 18: Ad-mediated enhancement in the cytotoxicity of a plasmid pULI100 in breast tumor and bone marrow cells. MDA-MB-231 (500), and CD34+ bone marrow cells (1000) were transfected with pULI100 plasmid in the absence and presence of dl312 (10 pfu/cell) and lipofectamine. Cell survival was estimated for MDA-MB-231 by a calorimetric assay and for CD34+ bone marrow cells by the colony forming assay described in Example 15. Uninfected cells were treated as 100% survival. Results of MDA-MB-231 cells are shown by solid bars, and bone marrow cells by the hatched bars. Results shown are the average of the triplicate determinations.

Figures 19A and 19B: AdWtp53-induced apoptosis in human breast cancer cells. Shown are cells infected with AdControl (Figure 19A) and with AdWtp53 (Figure 19B).

Figure 20: Effect of AdWtp53 injection on the growth of MDA-MB-231 xenografts in nude mice. MDA-MB-231 cells were injected subcutaneously in nude mice. 2 weeks after injection (day 0) tumors were given weekly injections of either AdControl (10⁹ pfus) or AdWtp53 (10⁹ pfus). Tumor sizes were measured on the days shown in Figure 20 and are represented by a solid bar for AdControl and a hatched bar for AdWtp53 infected tumors.

Figures 21A and 21B: Nude Mice Photos. Figure 19A shows a photograph of the animal that received an injection of AdWtp53, indicating that the tumor size disappeared completely. Figure 19B shows a photograph of the animal that received an injection of AdControl, indicating that the tumor size increased further.

Figur 22: β -gal Expression in MDA-MB-231 and Human Bone Marrow Cells Following Transfection with a Plasmid CMV β -gal in th Absence and Presence of dl312 and lipofectamine. The

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dotted bars represent the enzyme activity obtained in MDA-MB-231 cells and solid bars in CD34+ cells from G-CSF-mobilized human peripheral blood cells. Results shown are the mean of the triplicate determinations \pm S.D.

Figure 23: Cell Cycle Analysis of Human Aortic Vascular Smooth Muscle Cells Infected with AdWTp53 and AdWAF1. 2×10^5 of human aorta vascular smooth muscle cells were infected with AdWTp53, AdWAF1 and AdControl (50 pfu/cell) for 48 hours and subjected to cell cycle analysis. Results shown are the cell cycle analysis of uninfected cells (Panel A), AdControl-infected cells (Panel B), AdWAF1-infected cells (Panel C) and AdWTp53-infected cells (Panel D). The arrow indicates the population of cells in G1 subgroup. Changes in the percentage of cells exposed to various doses of (1, 10 and 50 pfu/cell) AdWTp53 (■) and AdWAF1 (●) in G1 phase (Panel E), S phase (Panel F) and G2/M phase (Panel G) are shown.

Figure 24: Cytotoxicity of AdWTp53, AdWAF1 and AdControl to human aortic vascular smooth muscle cells. The cytotoxicity of each adenovirus on human aortic vascular smooth muscle cells (250/well) was determined in triplicate on each wells of 96 well plates, exposed to AdWTp53 (■), AdWAF1 (●) and AdControl (□) of up to 40000 pfu/cell and after 7 days the number of cells was analyzed by colorimetric assay. Values shown are mean \pm S.E.

Figure 25: Figure 25 sets forth a schematic diagram of Adp27.

Figure 26: Figure 26 shows Adp27-mediated p27 Expression in Human Breast Cancer Cells.

Figures 27A and 27B: Effect of Adp27-mediated expression of p27 on DNA Cell Cycle Histograms.

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Figure 28: Effect of Adp27 Infection on Apoptosis.

Figure 29: Figure 29 sets forth a schematic diagram of AdCD.

Figures 30A and 30B: Figure 30A sets forth AdCD-mediated cytotoxicity of MCF-7 cells in the presence of 5-FC. Figure 30B sets forth AdCD-mediated cytotoxicity of MDA-MD-231 in the presence of 5-FC.

Figure 31: Figure 31 sets forth a schematic diagram of Adp16.

Figure 32: Figure 32 shows Adp16-mediated p16 expression in various cell cancer lines.

Figure 33: Figure 33 sets forth a schematic diagram of AdTAM67.

Figure 34: Figure 34 sets forth a schematic diagram of AdB7-1.

Figure 35: Figure 35 sets forth a schematic diagram of AdB7-2.

Figure 36: Figure 36 depicts AdWTp53-mediated Expression.

Figure 37: Figure 37 illustrates the effect of AdWTp53 Infection on Cell Cycle.

Figure 38: Figure 38 sets forth the Effects of AdWTp53 on Apoptosis.

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DETAILED DESCRIPTION OF THE INVENTION:

The present invention provides novel methods for the construction of recombinant adenoviral vectors capable of expressing human cDNAs. Specific non-limiting examples of such human cDNAs are wild-type p53, WAF1/Cip1/p21, p27/kip1, E. coli cytosine deaminase, wild-type p16, TAM 67 (a jun/fos dominant negative mutant) and B7-1 and B7-2.

The present invention affords a novel method for the construction of the adenoviral vectors by the addition of a second ClaI site for utilization in excising the 5' end of the adenoviral genome.

The present invention additionally provides a method of inhibiting the proliferation of cells. This method comprises contacting the cells with an adenoviral vector, capable of expressing human cDNAs, in an amount effective to inhibit cell proliferation. Particular non-limiting examples of such vectors are AdWtp53, AdWAF1, Adp27, AdCD, Adp16, AdTAM67, AdB7-1 and AdB7-2.

Further, the invention provides a method of inhibiting the cell cycle of proliferating cells. This method comprises contacting the cells with an adenoviral vector, capable of expressing human cDNAs, in an amount effective to inhibit DNA synthesis.

The present invention also provides a method of eradicating cancer cells by contacting the cells with an adenoviral vector, capable of expressing human cDNAs, in an amount effective to eradicate the cancer cells.

The recombinant adenoviral vector as described herein can be used and engineered to contain and express other genes (i.e. cDNAs) that may be useful for eradicating tumor cells in which the vector is expressed via the toxic effects of the expressed genes. Non-limiting examples of other cDNAs that can be used in the adenovirus vectors of the invention are HSVTK, No-synthetase, GADD 45, p15, mdm2, Rb, BAX, IL2, GMCF, p53-antisense, Her/Neu2 antisense, and Erb4 antisense.

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0 The present invention also provides for a method of
treating a subject afflicted with a tumor that has shown
resistance to drugs which comprises contacting the tumor
with an effective amount of a recombinant adenovirus capable
of expressing human cDNAs so as to inhibit proliferation of
5 the tumor cells.

A preferred recombinant adenovirus vector expressing
human wild-type p53 cDNA is AdWtp53. AdWtp53 possesses a
human-wild type p53 expression cassette consisting of a left
10 inverted terminal repeat, an origin of replication,
encapsidation signals, an Ela enhancer derived from
adenovirus type 5, a 1.7 kb human wild-type p53 cDNA, and an
SV40 maturation signal.

The amount of the recombinant adenovirus vector
expressing human wild-type cDNA effective to inhibit cell
15 proliferation of actively proliferating cells will vary
according to the cell type. Maximal inhibition of cell
proliferation by the recombinant adenovirus vector is
achieved on cancer cells that are either deficient in the
p53 protein or those that express a mutant p53 protein.

20 The present invention further provides a method of
treating a subject afflicted with a tumor which comprises
contacting the tumor with an amount of a recombinant
adenovirus vector capable of expressing human cDNAs which is
administered to the subject previous to, simultaneously, or
25 subsequent to, administration of a chemotherapeutic agent or
to an amount of irradiation effective to treat the tumor.
Examples of chemotherapeutic agents are known to those
skilled in the art and include, but are not limited to,
bleomycin, mitomycin, cyclophosphamide, doxorubicin,
30 paclitaxel, and cisplatin (See El-Deiry, et al., Cell,
75:817-825 (1993)).

In one embodiment of the invention, the recombinant
adenovirus vector containing a gene or cDNA of interest is
administered in a pharmaceutically acceptable carrier. A
35 pharmaceutically acceptable carrier encompasses any of the

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standard pharmaceutical carriers such as sterile solution, tablets, coated tablets and capsules. Such carriers may typically contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stensic acid, talc, vegetable fats or olis, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives and other ingredients.

The administration of the composition may be effected by any of the well known methods, including but not limited to, oral, intravenous, intramuscular, and subcutaneous administration. The preferred method of administration of the composition is local, i.e. at the site of the tumor.

In the practice of the method of this invention the amount of a recombinant adenovirus vector capable of expressing human cDNAs incorporated in the composition may vary widely. Methods for determining the precise amount depend upon the subject being treated, the specific pharmaceutical carrier, the route of administration being employed, the frequency with which the compound is to be administered, and whether the composition is administered in conjunction with a chemotherapeutic agent and/or irradiation treatment. The preferred amount of the vectors which may be administered for effective inhibition of proliferation of cells ranges from 10^8 to 10^{10} pfu/tumor.

The invention provides a novel method for the construction of adenoviral vectors. This method is not hampered by the problems which presently exist in the construction of adenoviral vectors. The invention provides a construction technique whereby an additional ClaI site is introduced at the 5' end of the adenovirus genome. The introduction of the second ClaI site at the 5' end of the adenovirus genome greatly reduces the chances of obtaining undigested genome as only one of the two ClaI sites will have to be cut to prevent the production of the non-recombinant background infectious virions. Moreover, because the novel adenovirus vectors will be made using

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viral genomic DNA as the starting point, there is no need to utilize a plasmid based vector.

In one embodiment of the invention, an adenovirus vector expressing human wild-type p53 cDNA (AdWtp53) was constructed. In the AdWtp53 genome, an additional ClaI restriction site was generated, thus providing two(2) ClaI restriction sites for excising the 5' end of the adenovirus genome. AdWtp53 was used as a parental adenovirus genome to generate future recombinant adenoviruses. In a further embodiment of the invention, a recombinant adenovirus which contains WAF1/Cip1 cDNA (AdWAF1) was constructed.

Utilizing two recombinant adenoviruses expressing wild-type p53 and WAF1/Cip1 cDNAs, it was determined that p53-mediated effects on cell cycle arrest are associated with the WAF1/Cip1 expression, and WAF1/Cip1 overexpression in the absence of p53 overexpression fails to induce apoptosis. Thus, the effects of p53 on apoptosis apparently require the cooperation of other signal transduction agents besides WAF1/Cip1.

In another embodiment of the invention, an adenovirus vector expressing human WAF1/Cip1/p21 cDNA (AdWAF1) was constructed. Recently, radiation-induced p53 effects were shown to be associated with WAF1/Cip1 induction (El-Deiry, et al., Cancer Res., 54:1169-1174 (1994)). However, whether WAF1/Cip1 overexpression in the absence of other p53-mediated signals induces apoptosis was not clear. However, the construction of the adenovirus vectors AdWtp52 and AdWAF1 of the present invention, which can both induce WAF1/Cip1 gene overexpression, allowed the direct investigation of the role of WAF1/Cip1 induction independent of p53 overexpression. It appears that AdWtp53-mediated p53 overexpression induces expression of WAF1/Cip1 in cells resulting in cell cycle arrest in all cells studied and apoptosis in those cells lacking expression of endogenous p53 or cells expressing mutant p53. In contrast, AdWAF1-mediated WAF1/Cip1 expression in infected cells resulted in

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cell cycle arrest without inducing apoptosis. Thus, the cytotoxic effects of wild type p53 involves additional factors besides WAF1/Cip1. It is already known that p53 overexpression can transcriptionally activate several genes, including MDM2 (Katayose, et al., Clin. Cancer Res. (Submitted 1995); Momand, et al. Cell 69:1237-1245 (1992)) GADD45 (Smith, et al. Science 266, 1376-1379 (1994)). The p53-mediated apoptotic pathway could involve induction of these or previously unidentified genes.

It is also important to note that AdWTP53 and AdWAF1 infection produces marked differences in cell cycle arrest. Infection with AdWTP53 resulted in a decline in the S phase and an increase in the proportion of G2+M. In contrast, AdWAF1 infection caused a marked increase in the proportion of cells in the G1 phase and a decline in S phase cells. These results indicated that WAF1/Cip1 overexpression causes cell cycle arrest at G1/S checkpoint while p53 can result in cell cycle arrest at G1/S and G2+M checkpoints. While the effects of both of these vectors on G1/S arrest can be explained by the WAF1/Cip1-mediated inhibition of CDK kinases, resulting in dephosphorylation of Rb protein (Dulic, et al., Cell 76:1013-1023 (1994); Michalovitz, et al., Cell 62:671-680 (1990); Ewen, M. E., Cancer Metastasis Rev. 13:45-66 (1994)) the mechanisms of AdWTP53-mediated accumulation of G2 + M cells remain unknown. It may be speculated that the cells arrested in G2 + M stage (in response to p53 overexpression) are perhaps more prone to undergo apoptosis, while G1/S arrested cells (in response to WAF1/Cip1 induction) remained viable for at least limited time period.

The differential effects of p53 and WAF1/Cip1 overexpression on cell cycle arrest and apoptosis were translated in the overall cytotoxic effects of AdWTP53 and AdWAF1. Thus, cells (H-358 (lung cancer cell line) and MDA-MB-231 (breast cancer cell line)) which undergo both cell cycle arrest and apoptosis (in response to p53

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overexpression) were more sensitive to cytotoxic effects of AdWtp53 as compared to AdWAF1; and cells (MCF-7, NMECs) which exhibit only growth arrest (by p53 or WAF1/Cip1 overexpression) were less sensitive to the cytotoxic effects of AdWtp53 and AdWAF1. Although the endogenous p53 status appears to play a role in determining the overall cytotoxicity of AdWtp53 and AdWAF1, it is important to note that the consequences of p53 and WAF1/Cip1 overexpression could be also dependent upon the amounts of p53 and WAF1/Cip1 produced in different cells, differential stability of these proteins in different cells, differential localization of these proteins within the cell, the ability or inability of these proteins to interact with other cellular factors or other downstream signals, and the absence or presence of other effector molecules (Haldar, et al., Cancer Res. 54:2095-2097 (1994)). It is also possible that the AdWtp53-mediated effects are not necessarily at the transcriptional level but perhaps involve other post transcriptional regulation as previously suggested (Caelles, et al., Nature 370:220-223 (1994)) and could also depend upon the cell's DNA repair ability (Modrich, P., Science 266:1959-1960 (1994)).

Independent of the mechanisms of the differential cytotoxicities, both AdWtp53 and AdWAF1 have clinical utility. The AdWtp53 vector may be clinically useful in tumors expressing mutant p53, which includes many human cancers (Nigro, et al., Nature 342:705-708 (1989); Takahashi, et al., Science 246:491-494 (1989); Srivastava, et al., Nature 348:747-749 (1990); Katayose, et al., Clin. Cancer Res. (Submitted 1995); Liu, et al., Cancer Res. 54:3662-3667 (1994); Fujiwara, et al., Cancer Res. 54:2287-2291 (1994)). The AdWAF1 vector may be useful in clinical settings wherein growth arrest of cells is an effective treatment.

The present invention also provides for the use of the novel AdWtp53 vector in order to elucidate the role of p53

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in inducing growth inhibition and apoptosis in vascular smooth muscle cells. Specifically, the role of p53 was analyzed by observing the effects of AdWtp53 on the proliferation and apoptosis of cultured human aortic vascular smooth muscle cells (AoVSMC) (See Example 25). In addition, the AdWAF1 vector of the present invention, expressing p53-inducible p21 cyclin-dependent kinase inhibitor, was also used to further elucidate the role of p53 in cancer.

The results of these studies have shown that adenovirus vector expressing p53 induced marked cytotoxicity in primary cultured AoVSMC. This cytotoxicity was associated with cell cycle arrest in G1 and G2/M boundary, accumulation of cells in G1 subgroup and perhaps apoptosis. Examining the mechanisms of p53-mediated cytotoxicity to AoVSCM is an important issue because as to date, there is no report to indicate that p53 induces apoptosis in normal cells, including vascular smooth muscle cells. This examination is made possible by the use of the vectors of the present invention. In this context, the involvement of p21 was studied by examining the overexpression of p21 in AoVSMC infected with either AdWtp53 or AdWAF1. Since AdWaf1 was about 200 times less toxic than AdWtp53, it is therefore unlikely that induction of p21 due to p53 overexpression mediated the cytotoxicity of AdWtp53 to AoVSMC. Further, the data herein shows that AdWtp53 induced not only G1 arrest but also G2/M arrest in AoVSMC, whereas AdWAF1 only induced G1 arrest.

The present invention further provides for the use of adenoviral vectors in cancer gene therapy. The presence of mutated p53 is widespread in different human cancers. Thus, reconstituting tumor suppressor p53 gene expression by adenoviral vectors is an attractive strategy for gene therapy. Since the adenovirus enters human epithelial cells with an efficient low-pH endosomes mediated endocytosis (Seth, et al., (1986) Pathway of adenovirus entry into

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cells, p. 191-195 In Colwell, R. and Lonberg-Holm, K. (ed.), Virus attachment and entry into cells, American Society for Microbiology, Washington, D.C.; Rosenfeld, et al., Hum. Gene Ther., 5:331-342 (1994)), tumors of mammary epithelial origin will be especially amenable to treatment by AdWtp53.

The present invention demonstrates that normal mammary epithelial cells are resistant to apoptosis by AdWtp53, while tumor cells null for p53, or expressing mutant p53, readily undergo apoptosis. These results demonstrate a specificity to AdWtp53-mediated eradication of tumor cells, lending further support for the utilization of adenoviral vectors in gene therapy.

Human adenoviruses have been used previously in gene transfer techniques in eucaryotic cells either by infecting the cells with a recombinant adenovirus expressing the transgene, or by adenovirus-mediated transfection of the plasmid DNAs.

The present invention shows that breast tumor cells are a much better target for Ad-mediated gene transfer as compared to bone marrow cells. The present invention also indicates that adenoviruses, in combination with appropriate toxic genes, can kill breast tumor cells while sparing bone marrow cells.

The present invention allows a comparison of the infectability using a replication-deficient adenovirus expressing β -galactosidase (β -gal gene). In addition, the invention can be used to determine or measure the relative cytotoxicity of the adenoviral vector encoding the catalytic domain of the Pseudomonas exotoxin gene.

The vectors of the invention cause an increase in the infection and transfection efficiencies of plasmid DNA in the presence of adenovirus in human breast tumor and bone marrow cells. Following infection of breast tumor cells with an adenovirus expressing β -galactosidase gene, high levels of β -galactosidase activity were observed, while normal bone marrow cells expressed about 500-fold less β -

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galactosidase activity. A recombinant adenovirus expressing human wild-type p53 protein (AdWtp53) was highly cytotoxic to breast tumor cells (100% of cell killing observed by 100 pfu/cell); while bone marrow cells were relatively resistant to the cytotoxic effects of AdWtp53 (less than 10% cell killing observed by 1000 pfu/cell).

One main reason that adenoviruses are effective for delivering genes to breast tumor cells as compared to bone marrow cells may relate to the high number of adenovirus receptors present on the surface of the cell. Therefore, other tumors found to have high levels of adenoviral receptors could be used in accordance with the invention. Human breast tumor cells tested were found to have fairly high number of adenovirus receptors (in the range of $5-10 \times 10^3$) while bone marrow cells are relatively deficient in cell surface adenovirus receptors (less than 5×10^2 /cell). Because adenovirus-mediated gene transfer requires the receptor-mediated uptake of adenovirus into the cells, breast cancer cells expressing adenoviral receptors would be a better target for adenoviruses than the adenovirus receptor deficient bone marrow cells.

In addition, the efficiency of adenoviral-mediated transfections may be enhanced by using a replication-deficient adenovirus mutant dl312, and even further enhanced by the addition of a liposome, such as lipofectamine and other mono and polycationic lipids. The present disclosure indicates that human bone marrow cells treated under the same conditions expressed very low levels of the transfected β -galactosidase DNA. It was also determined that transfection of cells with plasmid DNA expressing Pseudomonas exotoxin gene in the presence of dl312 and lipofectamine resulted in greater than 90% breast tumor cell killing, while human bone marrow CD34+ cells were at least 500-times more resistant to this treatment.

Since infection of breast tumor cells with a recombinant adenovirus expressing a toxic transgene (e.g., human WT p53)

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or transfecting with a toxin gene (Pseudomonas exotoxin) and in the presence of adenovirus resulted in marked cytotoxicity in breast tumor cells, with little or no toxicity to bone marrow cells, human adenoviruses are useful for cancer gene therapy and for removing or "purging" cancer cells from bone marrow. Human adenoviral vectors are also useful in cancer gene therapy for the treatment of lung, prostate, and liver cancer cells, and may be useful in the treatment of leukemia.

Adenovirus vectors which express a toxic gene may be used in gene therapy, or, alternatively, a replication-deficient adenovirus in combination with plasmid DNAs coding for a toxin gene may be used. The present disclosure indicates that since the use of both results in at least 100-fold increase in cytotoxicity to breast cancer cells compared to bone marrow cells, this approach offers a fairly wide efficacy window for purging. The protocols for human bone marrow purging using recombinant adenovirus vectors appear to be simple and effective. Recombinant adenoviruses for use in the protocols are replication-deficient, which pose few problems to the bone marrow cells. Moreover, if adenoviruses are used in combination with toxin genes, much lower dosage of adenoviruses will be required, thus making the bone marrow purging protocol even more safe. Because breast cancer cell contamination in human bone marrow which is to be used for marrow transplantation is potentially a serious problem, the adenoviral vectors of the present invention are necessary and useful tools for purging bone marrow cells of such contaminating tumor cells.

Adenoviral vectors may also be employed in the eradication of cancer cells. The effects of Adp53 on human breast tumors (derived from MDA-MB-231 cells) grown as xenografts in nude mice was investigated. MDA-MB-231 cells were injected subcutaneously in nude mice and two weeks later when the tumors were palpable, they were injected with AdWtp53 (10^9 pfus) or a control adenovirus (AdControl) (10^9

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pfus). Tumors which received a control virus continued to grow over the next 14-21 days, while tumors which received AdWtp53 exhibited inhibition of subsequent tumor growth. Tumors of nude mice that were injected with AdWtp53 disappeared completely over the course of the treatment (See Figure 21A), while the tumors of the nude mice that were injected with AdControl (adenovirus only) increased in size (See Figure 21B). These results thus confirm that the adenoviral vectors of the present invention would be extremely useful in cancer gene therapy. A preferred mode of administration is by direct injection. Therefore, the adenoviral vectors of the invention will be useful for the eradication of cancer cells by contacting the cancer cells of the tumor with an amount of the adenoviral vector sufficient for the eradication of the cancer cells. A particular example of such a vector for use in this treatment is AdWtp53. The adenoviral vector may also be administered to a subject previous to, simultaneously, or subsequent to, the administration of a chemotherapeutic agent or an amount of irradiation effective to eradicate the cancer cells.

The adenoviral vectors are also useful in the prevention of the development of cancer cells in those subjects who are at risk of developing cancer. The preventative treatment involves the administration of an adenoviral vector expressing the desired DNA which eradicates the cancer, via the toxic effects of the expressed genes, in an amount effective to prevent or inhibit the development of cancerous cells.

The present invention also provides for the construction and use of an adenoviral vector capable of expressing p16/INK4 kinase inhibitor. (See Example 5) It has been suggested that, for p16/INK4, mediated growth arrest is tightly associated with the status of Rb protein phosphorylation. In order to test the idea how p16 and Rb proteins mediated cell cycle arrest can be controlled by the

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endogenous background of p16 and Rb, the effects of adenoviral-mediated expression of p16 and Rb protein in several cell lines with different intrinsic status of p16 and Rb protein were herein investigated. (See Example 17)

As shown in Figure 32, in each cell line infection by Adp16 led to an increased expression of p16 protein. However, in cells infected with a control adenovirus, no increase in the basal level of p16 was observed.

Similarly when cells were infected with AdRb, high level protein expression of Rb protein was observed in each cell line. However, the expression of a control protein Actin was not altered following any adenoviral infection. These results indicate that Adp16-mediated expression of p16, and AdRb-mediated expression of Rb protein was independent of the endogenous status of either p16 or Rb.

The effects of p16 and Rb expression on cell cycle progression was investigated in cells with different endogenous p16 and Rb status. Different cells were infected with different doses (1-200 pfu/cell) of either Adp16, AdRb or AdControl, and 24 hours later analyzed for cell cycle distribution. Following Adp16 infection, in each cell line expressing endogenous wild type Rb protein (MCF-7, MDA-MB-231), an increase in cells in G1 and a decrease in cell number in S phase was observed, indicating that the cells were arrested at G1/S. The accumulation of cells in G1 phase was dependent upon the concentration of Adp16 used. However, in cells expressing non-functional form of Rb protein, Adp16 failed to induce cell cycle arrest. The effect of Adp16 on cell cycle arrest was not dependent on the endogenous status of p16. In each cell line used in this study (except those defective for Rb), Adp16 induced a strong cell cycle arrest, which was dependent on the dose of Adp16 used.

The present invention also provides for the construction and use of an adenoviral vector capable of expressing p27/kip1, a cell cycle inhibitor that is involved in the

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signal transducing effects of TGF β . (See Example 3) The recombinant adenoviral vector Adp27 of the present invention was used to study the relationship between p27/kip1 effects on cell growth, cell cycle, cyclin kinases and apoptosis. It is herein shown that p27/kip1 expression can regulate the cell cycle at both G1/S and G2/M check points, and that these effects are associated with the inhibition of cdk2 kinase and cdc2 cyclin B1-associated kinases. However, inhibition of these kinases did not result in cellular apoptosis, indicating the dissociation of cell cycle arrest at G1/S and G2/M phase from apoptosis. Thus, while cell cycle progression can be linked with cyclin kinase activities, its relationship with apoptosis is much more complex.

The present invention further provides for adenoviral vectors for use in treating drug resistant cancers. The cytotoxic effects of AdWtp53 in two drug resistant breast cancer cells; adriamycin resistant human breast cancer MCF-7 cells (MCVF-Adr) and mitoxantrone resistant MCF-7 cells, were herein investigated. (See Example 25) Following infection by AdWtp53, all the cell lines expressed high levels of p53 protein. However, MCF-Adr and MCF-Mito cells were much more sensitive to killing by AdWtp53 as compared to the parental MCF-7 cells. (See Table 5) The AdWtp53-mediated cytotoxicity in drug resistant cells was associated with cell cycle arrest (in G1/S and G2/M phase); inhibition of at least two cyclin kinase cdk2 and cdc2 cyclin B1-associated kinases, and apoptosis. Thus, the use of the novel adenoviral vectors of the present satisfies a needed requirement for approaches for treatment of drug resistant cancers.

The present invention further provides for adenoviral vector constructs that would be useful in exploring the clinical utility of suicidal enzymes for the gene therapy of breast cancer. While adenoviral vectors have many attractive features, a key problem with adenoviral vectors

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is that they can only infect a small population of cancer cells within a tumor mass, leaving many of the cells uninfected. Thus, there is a need to develop adenoviral vectors which should induce cytotoxicity across the whole tumor should adenovirus infect only a small number of tumor cells. One approach would be to use adenoviral vectors which under certain circumstances can be made to produce cytotoxic products which are smaller in size and hence will have opportunities to escape the cells and kill the uninfected cells; or diffuse across cell boundaries (e.g. through gap junctions) providing bystander effect. One such gene is E. coli deaminase which can convert a pro-drug 5-fluoro cytosine into a toxic species 5-fluoro uracil. Thus, the present invention provides for the construction and use of an adenoviral vector capable of expressing E. coli cytosine deaminase. (See Example 4) It is shown herein that the presence of 5-FC, AdCD is extremely cytotoxic to MDA-MB-231 and MCF-7 breast cancer cells, and show that bystander effects play a role in overall cytotoxicity of AdCD. Further, investigation of the cytotoxic effects of AdCD in vivo in the presence of 5-FC in human breast tumors grown as xenografts in nude mice. Thus, AdCD has potential clinical applications for the treatment of breast cancer. Because adenoviruses can express the transgenes to very high levels, coupled with the bystander effects of the suicidal genes, AdCD has advantages over retroviruses expressing E. coli CD.

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EXAMPLES

The Examples herein are meant to exemplify the various aspects of carrying out the invention and are not intended to limit the scope of the invention in any way. The Examples do not include detailed descriptions for conventional methods employed in the construction of vectors, the insertion of cDNA into such vectors, or the introduction of the resulting vectors into the appropriate host. Such methods are well known to those skilled in the art and are described in numerous publications, including Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA, (1989).

EXAMPLE 1**Construction of an Adenovirus Vector Coding For the Expression of Human Wild-Type p53 Protein (AdWtp53).**

Homologous recombination between a shuttle vector pDK10, containing an expression cassette of human wild type p53 cDNA and the adenovirus genome cloned in a plasmid pJM17 generated an adenovirus clone in which the adenovirus E1 region was replaced by the wild type p53 cDNA expression cassette.

Plasmid AdWtp53 was constructed using co-transfection of shuttle vector pDK10 containing the wild type p53 expression cassette and a plasmid pJM17 containing the adenovirus type 5 genome. pJM17 may be obtained from Microbix Biosystems, Inc., Toronto, Ontario Canada. pDK10 was deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland on February 17, 1995 under ATCC Accession No. 97064.

The shuttle vector pDK10 was constructed by inserting the cytomegalovirus (CMV) immediate early promotor and enhancer, a 1.7-kilobase XbaI fragment of human p53 cDNA (See Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)), the SV40 small T intron, and an SV40 polyadenylation signal into

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the ClaI site of plasmid pXCX2 (See Graham, et al. (1991) Manipulation of adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene transfer and expression protocols, Humana Press, Clifton, New Jersey).

Figure 1 shows the schematic diagram of AdWtp53. The 5' end of the genome contains the AdWtp53 expression cassette (10.3 mu) followed by the rest of the adenovirus genome. The key elements of the expression cassette of AdWtp53 include the left inverted terminal repeat (ITR), the adenoviral origin of replication, encapsidation signal, the E1a enhancer, the CMV immediate early promotor, the human wild type p53 cDNA and SV40 polyadenylation signal.

Plasmid pDK10 was co-transfected with pJM17 (See McGrory, et al., Virology, 163:614-617 (1988)) (kindly provided by F. Graham, McMaster Univ., Hamilton, Ontario) into the transformed human embryonic kidney cell line 293 (ATCC Accession No. CRL1573) by calcium phosphate mediated gene transfer technique (See Graham, et al. (1991) Manipulation of adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene transfer and expression protocols, Humana Press, Clifton, New Jersey; and Gilardi, et al., FEBS Lett., 267:60-62 (1990) (Gibco BRL, Gaithersburg, MD)). The day following transfection, the medium was replaced with a 1xMEM (Gibco BRL) containing 1% sea plaque agarose gel (FMC, Rockland, ME) and 10% fetal bovine serum (FBS) (Gibco BRL) and the cells were incubated at 37°C. Every five days 2 ml of MEM containing 1% sea plaque agarose gel and 10% FBS was added to the top of the cells until plaques were observed. Isolated plaques were picked and subjected to another cycle of infection in 293 cells as described previously (See Graham, et al. (1991) Manipulation of adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene transfer and expression protocols, Humana Press, Clifton, New Jersey; and Gilardi, et al., FEBS Lett., 267:60-62 (1990)).

Purified recombinant AdWtp53 was assayed for the absence of E1a and the presence of p53 sequences using the

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polymerase chain reaction (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). Briefly, 24 hours following infection with adenoviral vector, cell lysates were prepared using guanidine thiocyanate solution, and aliquots were obtained for use in the PCR analysis. In order to determine the absence of Ela nucleotide sequences, the following primers were used:

5'-TCTTGAGTGCCAGCGAGTAG-3' (SEQ ID NO:1); and

5'-CAAGGTTTGGCATAGAAACC-3' (SEQ ID NO:2).

In order to determine the presence of p53 nucleotide sequences, the following primer was selected from exon seven of the p53 nucleotide sequence:

5'-GTTGGCTCTGACTGTACC-3' (SEQ ID NO:3)

and the following downstream primer

5'-GTTCCGTCCCAGTAGATTACC-3' (SEQ ID NO:4)

was selected from exon eight of the p53 nucleotide sequence.

This combination of primers allows for the differentiation of the PCR product of the endogenous genomic p53 gene from the viral-associated p53 gene. AdWtp53 was propagated in 293 cells grown in monolayers, purified by two cesium chloride density gradients, dialyzed against a buffer containing 10% glycerol, 1 mM MgCl₂, pH 7.5, and stored at -70°C as described by Seth, et al., J. Virol. 68:933-940 (1994). PCR analysis of the purified AdWtp53 indicated that it contained p53 cDNA but was devoid of Ela sequences.

Control adenovirus vectors used in this study were: Ad.RSVβgal, an adenovirus vector containing β-galactosidase gene (Stratford-Perricaudet, et al., J. Clin. Invest., 90:

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626-630 (1992)), and AdControl, a null adenovirus vector dl312 (Jones, et al., Cell, 17:683-689 (1979) (kindly provided by R. Crystal, NIH, Bethesda)

EXAMPLE 2

Construction of an Adenovirus Vector Coding For the Expression of Human WAF1/Cip1 Protein (AdWAF1).

AdWAF1 was constructed by homologous recombination between a shuttle vector containing WAF1 (p21 cyclin-dependent kinase inhibitor) cDNA expression cassette (pDK13, see Figure 8) and ClaI cut genomic DNA derived from AdWTp53 using a previously described procedure (See Graham, F. L. and Prevec, L. (1991) In *Gene transfer and expression protocols*, (Murray, E. J. ed.) pp. 109-128, Humana Press, Clifton, New Jersey) to purify adenovirus genomic DNA. pDK13 was deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland on February 17, 1995 under ATCC Accession No. 97063. Following co-transfection into the transformed human embryonic kidney cell line 293 (obtained from American Type Tissue Culture Collection under ATCC Accession No. CRL 1573), adenovirus plaques were isolated as described previously (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). The presence of WAF1 cDNA in AdWAF1 was confirmed by polymerase chain analysis using the following primers:

5'-AGTCTCAGTTTGTGTGTCTTA-3' (SEQ ID NO:5);

5'-GTGCCATCTGTTTACTTCTCA-3' (SEQ ID NO:6).

AdControl used in this study was a replication-deficient adenovirus dl312 (Jones, N. and Shenk, T., Cell, 17:683-689 (1979)) (kindly provided by T. Shenk, Princeton University, Princeton, NJ). Adenoviruses were propagated in 293 cells, purified by two cesium chloride density centrifugation, tittered and stored at -70°C as described previously

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(Katayose, et al., Clin. Cancer Res. (Submitted 1995); Seth, et al., J. Virol. 68:933-940 (1994)).

EXAMPLE 3

Construction of a Recombinant Adenoviral Vector Coding for the Expression of Human p27 Protein (Adp27).

Adp27 was constructed by co-transfection of a shuttle vector pCG1 containing the p27 expression cassette and 35 kb fragment derived from an adenovirus expressing p53 (AdWtp53). The shuttle vector pCG1 was constructed by inserting the human cytomegalovirus (CMV) immediate promoter and enhancer, a 1.7 kilobase XbI fragment of p27 cDNA into a null shuttle vector described previously. The two DNAs were cotransfected into the transformed human embryonic kidney cell line 293 (ATCC CRL1573) by calcium phosphate mediated gene transfer technique (Gibco BRL, Gaithersburg, MD). After 24 hours, the medium was replaced with a 1xMEM (Gibco BRL) containing 1% sea plaque agarose gel (FMC, Rockland, ME) and 10% fetal bovine serum (FBS) (Gibco BRL). Every five days, 2 ml of MEM containing 1% sea plaque agarose gel and 10% FBS was added to the top of the cells until plaques were observed. Isolated plaques were picked and subjected to another cycle of infection in 293 cells as described previously. Several plaques were screened for the presence of p27 sequences by polymerase chain reaction (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). PCR reactions were also performed for E1 and p53 sequences using sets of primers described previously. Figure 25 shows the schematic diagram of Adp27. The 5' end of the genome contains the Adp27 expression cassette (10.3 mu) followed by the rest of the adenoviral genome.

The key elements of the expression cassette of Adp27 include the left inverted terminal repeat (ITR), the adenoviral origin of replication, encapsidation signal, the Ela enhancer, the CMV immediate early promoter, the human

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wild-type p53 cDNA and SV40 polyadenylation signal. Adp27 has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

EXAMPLE 4

Construction of a Recombinant Adenoviral Vector Coding for the Expression of E. Coli Cytosine Deaminase (AdCD).

AdCD was constructed by co-transfection of a shuttle vector pPS1 containing the E. coli cytosine deaminase expression cassette and 35 kb fragment derived from ClaI cut adenoviral genomic DNA as previously described. Plasmid pPS1 was constructed by inserting the human cytomegalovirus (CMV) early promoter and enhancer, a 1.1 kilobase XbaI fragment of CD cDNA into a null shuttle vector. Co-transfection and isolation of viral plaques were performed by published methods. Several plaques were screened for the presence of CD sequences by polymerase chain reactions (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). PCR reactions were also performed for E1 using sets of primers described previously. Figure 29 shows the schematic diagram of AdCD. The 5' end of the genome contains the AdCD expression cassette (10.3 mu) followed by the rest of the adenoviral genome. The key elements of the expression cassette of AdCD include the left inverted terminal repeat (ITR), the adenoviral origin of replication, encapsidation signal, the Ela enhancer, the CMV immediate early promoter, E. coli CD gene and SV40 polyadenylation signal. AdCD has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

EXAMPLE 5

Construction of a Recombinant Adenoviral Vector Coding for the Expression of Human p16 (Adp16).

A recombinant adenovirus expressing human p16 (Adp16) was constructed by using homologous recombination methods

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described previously to construct other adenoviral vectors. In brief, a shuttle vector pCG2 containing the p16 expression cassette was co-transfected with a 35 kb fragment derived from ClaI cut adenoviral genomic DNA in transformed human kidney 293 cells. Adenoviral plaques were picked and subjected to another cycle of infection in 293 cells as described previously. Several plaques were screened for the presence of p16 sequences by polymerase chain reaction (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). PCR reactions were also performed for E1 as described previously. A schematic diagram of Adp16 is set forth in Figure 31. Adp16 has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

Similarly, the following adenoviral vectors were prepared as set forth above:

(1) AdTAM67 (See Figure 33 for the schematic diagram).

(2) AdB7-1 (See Figure 34 for the schematic diagram).

(3) AdB7-2 (See Figure 35 for the schematic diagram).

AdTAM67, AdB7-1 and Adb7-2 have been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

EXAMPLE 6

Preparation of Cell Culture

Breast cancer cell lines, MDA-MB-231 (ATCC Accession No. HTB26), MCF-7 (kindly provided by R. Buick, Univ. of Toronto), Adr^R MCF-7, adriamycin resistant MCF-7 cells and MCF-Mito, mitoxantrone resistant MCF-7 cells (Fairchild, et al., Cancer Res., 47:5141-5148 (1987)) were cultured in alpha minimal essential medium (MEM) (Gibco BRL) supplemented with 10 mM Hepes, 2mM glutamine, 0.1mM nonessential amino acids, 10% FBS, 1ng/ml epidermal growth

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factor (EGF) and 2 μ g/ml insulin.

H-358, a lung cancer cell line (kindly provided by J. Minna, NCI-Navy Medical Oncology Branch, Bethesda, MD), and MDA-MB-453 cells, a breast cancer cell line (ATCC Accession No. HTB131), were grown in RPMI containing 10% FBS. MDA-MB-157 (ATCC Accession No. HTB 24) a breast cancer cell line, was grown in IMEM (Gibco BRL) supplemented with 10% FBS and 0.5% Redu-Ser II (Upstate Biotechnology Inc, Lake Placid, NY).

Normal Mammary Epithelial Cells (NMECs) derived from reduction mammoplasties (CC-201 6, Clonetic Corp., San Diego, CA), and 184B5 cells, immortalized mammary epithelial cells (ATCC CRL10317) were cultured in Mammary Epithelial Basal Medium (MEBM, Clonetics, Corp.) supplemented with 1x vitamins, 0.5% FBS, 20 ng/ml EGF, 5 μ g/ml hydrocortisone and 52 μ g/ml bovine pituitary extract (Gudas, et al., Cell Growth Differ., 5:295-304 (1994)).

Immortalized MCF10 cells (kindly provided by S. Brooks, Michigan Cancer Foundation) were cultured in DMEM/F12 (Gibco BRL) supplemented with 2.5% horse serum (Gibco BRL) 10 mM Hepes (Calbiochem, La Jolla, CA), 2 mM glutamine (Biofluids, Rockville, MD), 0.1mM nonessential amino acids, (Gibco BRL) 20 ng/ml EGF (Upstate Biotechnology), 10 μ g/ml insulin (Boehringer Mannheim, Indianapolis, IN), 0.5 μ g/ml hydrocortisone.

293 cells, (ATCC Accession No. CRL 1573), an adenovirus transformed human embryonic kidney cell line was cultured in improved minimal essential medium (Biofluids) supplemented with 2 mM glutamine (Biofluids), 2.5 mcg/ml fungizone (Biofluids), 100U/ml penicillin, 100 mcg/ml streptomycin (100xPen-Strep, Biofluids) and 10% FBS.

AoVSMC (CC-2023, MyoPack-AOSMC, Clonetics Corps., San Diego, CA) were cultured in SmGM, based on the MCDB 131 formulation, and supplemented with 10ng/ml hEGF, 2 mg/ml hFGF, 0.39 ug/ml Dexamethazone, 5% FBS, 50 ug/ml Gentamicin and 50 ng/ml Amphotericin-B.

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EXAMPLE 7**(1) Effect of AdWtp53 on Cell Growth**

The effect of high level wild type p53 expression on the growth of cells having varying p53 status was determined using the following cell lines:

(1) H-358 lung cancer cells, which are devoid of p53 protein (p53 null) (See Takahashi, et al., Science, 246:491-494 (1989));

(2) MDA-MB-231 human breast cancer cells, which express mutant p53 protein (Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)); and

(3) MCF-7 human breast cancer cells which express wild-type p53 protein (Casey, et al., Oncogene, 6:1807-1811 (1991); Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)).

5×10^4 cells of each respective cell line were plated in each well of 6 well tissue culture dishes. After 24 hours, the cells were exposed to 10 pfu/cell of AdWtp53 or AdControl in medium containing 2% fetal bovine serum. After an incubation of 2 hours at 37°C, the serum concentration in the medium was raised to 10% and the incubations continued at 37°C. The cells were trypsinized on each day and counted using a hemacytometer. The cytotoxicity of the adenovirus vectors was assessed using a colorimetric assay as described previously (Skehan, P., J. Natl. Cancer Inst., 82:1107-1112 (1990)). Briefly, 500 cells were plated in each well of 96 well plates. 24 hours later, the cells were exposed to the appropriate cell growth medium. However, in order to facilitate a more efficient infection, the concentration of the serum (if it was a component of the growth medium) was reduced to 2%. Varying doses of adenovirus vectors were included in the incubation medium (several 5-fold dilutions). After a 2 hour incubation at 37°C, the serum concentration was increased to 10% and the cells were incubated for 7 days at 37°C. The cells were fixed by the addition of ice-cold 50% trichloroacetic acid (TCA), which was added onto the top of the medium in each well to a final

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concentration of 10%, the cells were incubated at 4°C for 1 hour, washed five times with water, and allowed to air dry. TCA-fixed cells were stained for 20 minutes with 0.4% (wt/vol) sulforhodamine B (Sigma, St. Louis, MO) dissolved in 1% acetic acid, followed by rinsing four times with 1% acetic acid. An O.D.₅₆₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and was used as a measure of cell number. The percent survival rates of cells exposed to adenovirus vectors were calculated by assuming the survival rate of uninfected cells to be 100%.

As shown in Figures 3A and 3B, infection of H-358 and MDA-MB-231 cells with AdWtp53 completely inhibited cell growth over the four day period examined. In both of these cell lines, the cell number was reduced by day 4 to levels less than half of that present at time 0. In contrast, MCF-7 cells continued to proliferate although at a slower rate than control cells (See Figure 3C). Figures 3A-3C demonstrate that the AdControl virus had very little effect on the growth of these cells.

These results suggested that infection by AdWtp53 had a more profound growth inhibitory effect on cells that were either deficient in p53 or expressed a mutant p53 than on cells that expressed wild-type p53. To confirm these observations, the effects of AdWtp53 on cancer cells that lack p53 expression (H-358, MDA-MB-157) (Takahashi, et al., Science, 246:491-494 (1989); Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)), cancer cells that expressed endogenous mutant p53 (MDA-MB-231, MDA-MB-453) (McGrory, et al., Virology, 163:614-617 (1988); Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)), cancer cells that expressed wild type p53 (MCF-7), and immortalized and normal cells that expressed wild type p53 (MCF-10, 184B5, NMECs) (J. Gudas, et al. - unpublished data) were investigated. Briefly, each cell line was exposed to increasing concentration of AdWtp53 for 7 days. As shown in Figure 4 and Table 1, cells that are null for expression of p53 were the most sensitive to

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the inhibitory effect of AdWtp53 (IC_{50} for H-358 and MDA157 cells were 0.17 and 0.3 pfu/cell respectively). Cells that express a mutant p53 protein were only slightly less sensitive to the growth inhibitory effects of AdWtp53 (IC_{50} for MDA-MB-231, and MDA-MB-453 were 0.4 and 0.7 pfu/cell respectively). In contrast, immortalized or normal cells that expressed wild type p53 were the most resistant to the cytotoxic effects of AdWtp53, with NMECs being the most resistant (IC_{50} for 184B5, MCF-10, MCF-7 and NMEC were 4.5, 5.5, 30 and 100 pfu/cell respectively) (See Table 1). Thus, cells that express wild type p53 were 5-250 times more resistant to the AdWtp53-mediated inhibitory effect on cell growth when compared with cells expressing no p53 or mutant p53.

(2) β -galactosidase Activity Following Ad.RSV β gal Infection

Because differences in the sensitivity of various cell lines to AdWtp53 could result from either reduced uptake and/or decreased transgene expression, the expression of an adenoviral vector containing the marker gene, β -galactosidase was examined in the following cell lines: NMEC, MCF-7, MCF-10, MDA-MB-453, MDA-MB-231, MDA-MB-157, MDA-MB-453, and H-358.

The cells were plated at a concentration of 2×10^4 cells in each well of a 96 well tissue culture plate. After 24 hours, the cells were exposed to various concentrations of Ad.RSV β gal (0.1-500 pfu/cell) in medium used by each respective cell line, however, the serum concentration (if required) was reduced to 2%. After a 2 hour incubation at $37^\circ C$, the serum concentration (if required) was raised to 10%, and the cells were then incubated at $37^\circ C$ for an additional 24 hours. The cells were washed three times with phosphate buffered saline pH 7.5 (PBS) and lysed in 50 μ l of 0.1M Tris pH 7.5 containing 0.1% Triton X-100. An aliquot (30 μ l) was assayed for β -galactosidase activity using a modified protocol (Stratford-Perricaudet, et al., J. Clin.

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Invest, 90:626-630 (1992)). Samples were transferred to each well of a 96 well plate and treated with 100 μ l of 20 mM Tris pH 7.5 containing 1 mM $MgCl_2$, 450 μ M β -mercaptoethanol, 150 μ M O-nitrophenyl- β -galactopyranoside. Incubations were performed at 37°C for 20 min. and the reaction stopped by the addition of 150 μ l/well of 1 M Na_2CO_3 . The optical density was determined at 420 nM. An O.D.₄₂₀ of 1 was defined as 1 unit of enzyme activity.

Following infection of each cell line at 20 pfu/cell, the enzyme activity in each cell line was in the range of 0.3-0.75 units (See Table 1). Moreover, as shown in Figure 5, each of the cell lines expressed high amounts of p53 when they were exposed to AdWtp53. Therefore, the differences in the sensitivity of killing effects of AdWtp53 cannot be explained by alteration in viral uptake and/or differential expression of the transgene.

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Table 1

Summary of the status of endogenous p53 in various cell lines, IC₅₀ values of AdWtp53 in each of the cell line, β -galactosidase (β -gal) activity in these cells after infecting with Ad.RSV β gal vector, and relative induction of WAF1/Cip1 protein expression following infection with AdWtp53.

Cell line	Endogenous	IC ₅₀	β -gal activity ^c	
			p53 status ^a	AdWtp53 ^b WAF1/Cip1 ^d
H-358	Null	0.17	0.75	105
MDA-MB-157	Null	0.30	0.54	83
MDA-MB-231	Mutant	0.4	0.66	154
MDA-MB-453	Mutant	0.70	0.58	71
MCF-7	Wild type	0.3030	0.30	2.3
184B5	Wild type	4.5	ND	2.4
MCF-10	Wild type	5.5	ND	7.5
NMECs	Wild type	100	0.731	1.2

^a The status of endogenous p53 in each cell line are taken from references 2, 16, 29, 32, 33 and J. Gudas, et al. (unpublished data) as described in the text.

^b These values are estimated from Figure 4.

^c β -galactosidase activity in each cell line was measured after infecting cells with 20 pfu/cell as described.

^d Fold-increase in the WAF1/Cip1 expression represent the AdWtp53-mediated (10 pfu/cell) increase in WAF1/Cip1 protein expression over the uninfected cells.

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EXAMPLE 8**Immunoprecipitation of p53 Protein in Cells Infected with AdWtp53**

Cells (1×10^6) were plated in 10 cm dishes and infected with AdWtp53 or AdControl for 24 hours as described in Example 6. Immunoprecipitations were performed using an anti-p53 antibody essentially as described (Seth, P., et al., Mol. Cell. Biol., 4:1528-1533 (1984)). Briefly, cells were incubated with 3 ml of methionine-free DMEM (Biofluids) containing 5% dialyzed fetal calf serum (Biofluids) and 100 μ Ci/ml of [35 S]methionine and [35 S]cysteine mixture (Express 35 S- 35 S-protein labeling mix, 1000 Ci/mmol, (NEN) for 2 hrs. Cells were washed with ice-cold PBS, and solubilized at 4°C in buffer A (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 0.1% Sodium deoxycholate (Sigma), 0.5% sodium lauryl sulfate (SDS) (Research Genetics, Huntsville, AL), 1 mM phenylmethyl sulfonylfluoride, 10 μ g/ml aprotinin, 1.0 μ g/ml leupeptin, 1.0 μ g/ml pepstatin (all protease inhibitors from Boehringer Mannheim). Aliquots of 500 μ l of 35 S-labeled lysates were incubated with a 1:50 dilution of anti-p53 monoclonal antibody PAb 1801 (Ab-2) (Oncogene Science, Uniondale, NY) at 4°C for 1 hour, after which 15 μ l of protein A/G agarose (Oncogene Science) was added and the incubations were continued for an additional 1 hour with rotation. Samples were then centrifuged at 5000 x g for 5 min, and the pellets were washed successively with buffer A, buffer A containing 1M NaCl, and finally with buffer A again. SDS-gel sample buffer (50 μ l) was added, and the samples heated for 5 min at 95°C to elute proteins from the immunoabsorbent. The tubes were centrifuged again at 5000 x g for 5 min to remove protein A/G agarose, and 20 μ l aliquots of protein samples were subjected to SDS-polyacrylamide gel electrophoresis. The gels were then dried and exposed to X-ray film as described previously (Seth, et al., Mol. Cell. Biol., 4:1528-1533 (1984)).

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EXAMPLE 9**Western Blot Analysis of p53, WAF1/Cip1 and mdm2 Proteins in Cells Infected with AdWtp53**

In an effort to determine the molecular mechanisms underlying the cytotoxicity of AdWtp53, the expression of two cellular proteins that could play a role in mediating the inhibitory effects of p53 at both the protein and RNA levels was examined (See Example 6). These included WAF1/Cip1, a gene which is induced in cells and inhibits cyclin kinase (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Fairchild, Cancer Res., 47:5141-5148 (1987)), and mdm2, another p53 inducible gene that can bind p53 and modulate its function (Gudas, et al., Cell Growth Differ., 5:295-304 (1994)).

To demonstrate the effects of AdWtp53 and mdm2 on WAF1/Cip1 protein expression, RNA was prepared from the following cell lines and used in the Western blot analysis:

(1) Null p53 cell lines:

- (a) MDA-MB-157
- (b) H-358

(2) Mutant p53 cell lines:

- (a) MDA-MB-231
- (b) MDA-MB-4533

(3) Wild-type p53:

- (a) MCF-7
- (b) MCF-10
- (c) 184B5
- (d) NMECs

Each respective cell line was plated in 6 cm tissue culture dishes at a concentration of 0.5×10^6 and incubated with AdWtp53 or AdControl for 24 hours as described in Example 2. The cells were then washed three times with ice-cold PBS, scraped and resuspended in 1 ml of 1x SDS-poly acrylamide gel electrophoresis buffer (62 mM Tris pH 6.8, 2 mM ethylenediaminetetraacetate (EDTA), 15%

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sucrose, 10% glycerol, 3% SDS, 0.7 M 2-mercaptoethanol) and boiled for 10 min. Equal amounts (15 or 50 μ g) of denatured protein were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose filters (Gudas, et al., Cell Growth Differ., 5:295-304 (1994)). Filters were blocked with Tris-buffered saline containing 5% dried milk and 0.1% Tween 20 (Sigma). Blots were probed with 4 μ g/ml of Ab-2; and Ab-6 for p53, 4 μ g/ml of EA 10 for WAF1/Cip1, 3 μ g/ml of IF2 for mdm2, with 3 μ g/ml of Actin (Ab-1) antibody. All antibodies were obtained from Oncogene Science. Following incubation with the primary antibodies, the blots were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with horseradish peroxidase, conjugated to secondary antibody, and the specific complex was detected by the enhanced chemiluminescence technique according to the manufacturer's directions (NEN).

As shown in Figure 5, the Western blot analysis demonstrates that low levels of endogenous p53 were detected in all cell lines examined except MDA-MB-157 and H-358. However, the level of p53 increased substantially (at least 10-fold) in each cell line following AdWTp53 infection (10 or 50 pfu/cell). In contrast, the amount of p53 increased little, if at all, above the endogenous p53 protein level in cells exposed to 50 pfu/cell of AdControl. Because cells exposed to either AdControl or AdWTp53 expressed similar levels of actin protein, (See Figure 5), increased levels of p53 following AdWTp53 infection can not be due to loading of different amounts of proteins or other non-specific mechanisms.

The induction of WAF1/Cip1 expression following AdWTp53 infection was also examined. As shown in Figure 5, there was little or no detectable basal level of WAF1/Cip1 in cells that did not express endogenous wild type p53 (MDA-MB-157, H-358) or in cells that expressed a mutant p53 (MDA-MB-231, MDA-MB-453), basal levels of WAF1/Cip1 were readily detected in cells that expressed endogenous wild type p53

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(MCF-7, MCF-10, 184B5, NMECs). While exposure of cells to 50 pfu/cell of AdControl did not affect the basal level of WAF1/Cip1 in any of these cells, exposure to 10 or 50 pfu/cell of AdWtp53 resulted in a marked increase in WAF1/Cip1 expression in most of the cell lines (See Figure 5). AdWtp53 infection resulted in greater than 70 fold increase in WAF1/Cip1 protein in cells that lacked endogenous p53 gene expression (MDA-MB-157, H-358) or expressed mutant p53 (MDA-MB-231, MDA-MB-453) (See Table 1). The magnitude of WAF1/Cip1 induction observed following AdWtp53 infection was lower in MCF-7, MCF-10, 184B5 and NMECs; cells that expressed endogenous wild type p53 (See Table 1).

mdm2 protein levels were also determined before and after AdWtp53 infection in each cell line. Basal levels of mdm2 protein were not detectable in cells that were null for p53 or contained mutant p53. Endogenous mdm2 protein bands of approximately 90 kDa and 57 kDa (Gudas, et al., Cell Growth Differ., 5:295-304 (1994); and J. Gudas, et al. - unpublished data) were readily detected in all cells expressing wild type p53, and no difference in the levels of either of mdm2 proteins were observed following infection of cells with AdControl vector. In contrast, following exposure to AdWtp53 at 10 or 50 pfu/cell, there was a marked increase in the levels of high and low molecular weight mdm2 proteins in all cell lines examined - except MCF-7 cells in which AdWtp53-mediated expression of the 57 Kd protein was minimal.

EXAMPLE 10

Northern Blot Analysis of WAF1/Cip1 and mdm2 in Cells Infected with AdWtp53.

To demonstrate the effect of AdWtp53 and mdm2 on WAF1/Cip1 total RNA expression, RNA was prepared from the following cell lines and used in the Northern blot analysis:

- (1) NMEC (normal mammary epithelial cells;

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- (2) MCF-10 (breast cancer cells);
- (3) MCF-7 (breast cancer cells);
- (4) MDA-MB-453 (breast cancer cells);
- (5) MDA-MB-231 (breast cancer cells); and
- (6) MDA-MB-157 (breast cancer cells).

Each respective cell line was plated in 15 cm tissue culture dishes at a concentration of 2×10^6 , and incubated with 10 pfu/cell of adenoviral vectors. After incubation for 24 hours at 37 °C, RNA was extracted by rinsing the cells three times with cold PBS and dissolving them in a 2 ml solution of guanidine isothiocyanate. RNA was purified by centrifugation over a 5.7 M cesium chloride cushion (See Gudas, et al., Cell Growth Differ., 5:295-304 (1994)), fractionated by electrophoresis in agarose gels containing formaldehyde, transferred to Magna NT filters, and cross-linked as described previously in Gudas, et al., Cell Growth Differ., 5:295-304 (1994). Following prehybridization, the filters were hybridized using a 2.1 kb fragment of WAF1/Cip1 or an 800 bp fragment from 36B4. Following hybridization the filters were washed and exposed to X-ray films, and autoradiographs developed as described (See Gudas, et al., Cell Growth Differ., 5:295-304 (1994)).

Since p53 is a DNA binding transcription factor (Skehan, et al., J. Natl. Cancer Inst., 82:1107-1112 (1990)), it was determined whether AdWtp53-mediated induction of WAF1/Cip1 protein was regulated at the level of RNA. The expression of WAF1/Cip1 mRNA was assessed by Northern blot analysis following infection of cells with either AdControl or AdWtp53. As shown in Figure 6, cells devoid of Wtp53 (MDA-MB-157) and cells expressing mutant p53 (MDA-MB-453, MDA-MB-231) had very low levels of WAF1/Cip1 mRNA after infection with AdControl. NMECs, MCF-10 and MCF-7 cells all contained endogenous wild-type p53 and expressed varying levels of WAF1/Cip1 mRNA expression following infection with AdControl. Following infection of AdWtp53, the WAF1/Cip1 mRNA levels in cell lines null for p53 or

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expressing mutant p53 were induced significantly; MDA-MB-453 showed a 7.4 fold increase, MDA-MB-231 showed a 21 fold increase, and MDA-MB-157 showed a 8.2 fold increase. However, when cells expressing wild type p53 were infected with AdWtp53, only MCF-7 cells showed a marked 6 fold increase in WAF1/Cip1 mRNA levels. MCF-10 showed only a 2 fold increase, and NMECs only a 1.2 fold increase in WAF1/Cip1 mRNA following infection with AdWtp53. The level of a control mRNA (36B4) was similar in cells infected with either AdControl or AdWtp53. Thus, the induction of WAF1/Cip1 proteins in cells following infection with AdWtp53 is mediated by an increase in WAF1/Cip1 mRNA.

It was also determined that NMECs, which are the most resistant to killing by AdWtp53, despite the expression of high levels of p53 following AdWtp53 infection, did not undergo apoptosis and showed the smallest increase (1.2 fold) in WAF1/Cip1 induction. Conversely, tumor cells deficient in wild-type p53 or expressing mutant p53 were quite sensitive to the cytotoxic effects of AdWtp53 and showed marked induction of WAF1/Cip1 RNA and protein. Therefore, AdWtp53-mediated cytotoxic effects appeared to be associated with the high expression of WAF1/Cip1. The WAF1/Cip1 gene has been shown to bind to cellular cyclin CDK kinase and thereby inhibit their function (Katayose, et al., Am. J. Physiol., 264: L100-L106 (1993); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Fairchild, et al., Cancer Res., 47:5141-5148 (1987)). This inhibition is manifested in turn by a decrease in the level of phosphorylation of the Rb protein (Seth, et al., Mol. Cell. Biol., 4:1528-1533 (1984)).

EXAMPLE 11

Detection of Nuclear DNA Fragmentation in Cells Infected with AdWtp53

To investigate whether the mechanisms of AdWtp53-mediated inhibition of cell growth involved programmed cell

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death (apoptosis), the effect of AdWtp53 on nucleosomal DNA fragmentation was determined after infection of MDA-MB-231 cells (which express endogenous mutant p53), H-358 cells (which are null for p53), and in MCF-7 and NMECs (both of which express endogenous wild type p53).

Each respective cell line was plated at a concentration of 2×10^6 cells in 10 cm tissue culture dishes and incubated with adenoviral vectors (50 pfu/cell) for 24 hours. Both adherent and floating cells were collected together and pelleted by centrifugation at $1800 \times g$ for 5 min (RT-6000B, Du Pont, Boston). Cell pellets were rinsed with cold PBS and low molecular weight DNA was prepared by a modified Hirt extraction method as described in Rosenfeld, et al., Hum. Gene Ther., 5:331-342 (1994). Briefly, pellets were lysed in 1 ml of 10 mM Tris, 10 mM EDTA disodium pH 7.4 (Research Genetics), 0.6% SDS (Research Genetics) and 0.2 mg/ml proteinase K (Boehringer Mannheim). Samples were incubated at 55°C for 5 hours, low molecular weight DNA was prepared by the Hirt extraction method as described in Rosenfeld, et al., Hum. Gene Ther., 5:331-342 (1994) and the evaluated by electrophoresis on a 2% agarose gel.

As shown in Figure 7, 24 hours following exposure of MDA-MB-231 cells to 50 pfu/cell of AdWtp53, several lower molecular weight DNA bands (DNA laddering of approximately 145 bp) in the range of 145-1050 bp were observed. This observation is characteristic of cells undergoing apoptosis. In contrast, exposure of MDA-MB-231 cells to AdControl or mock infection of these cells produced no detectable DNA laddering (See Figure 7). When MCF-7 cells or NMECs were exposed to AdWtp53 at 50 pfu/cell, no DNA laddering was observed (See Figure 7). Infection of MCF-7 cells with 1000 pfu/cell also did not demonstrate DNA laddering.

These results indicate that tumor cells null for p53 or expressing an endogenous mutant p53 undergo apoptosis following exposure to AdWtp53, while tumor cells or normal cells expressing wild type p53 are resistant to apoptosis.

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EXAMPLE 12**AdWtp53-mediated Synthesis of p53 protein**

To determine if AdWtp53 expresses p53 protein in tumor cells, the lung tumor cell line H-358, which lacks endogenous p53 (Takahashi, et al., Science, 246, 491-494 (1989)) was exposed to various concentrations of either AdControl or AdWtp53 for 24 hours. Following infection, immunoprecipitation of p53 was performed as described in Example 4. As shown in Figure 2A, there was no detectable p53 in H358 cells infected with AdControl. In contrast, p53 protein was easily detected by immunoprecipitation in cells infected with 1 pfu/cell of AdWtp53. Furthermore, the amount of immunoprecipitable p53 protein increased with increasing concentrations of AdWtp53 vector.

To investigate adenoviral-mediated p53 expression in breast tumor cells, several different mammary cell lines (MCF-7, MCF-10, Adr^R MCF-7, MDA-MB-231) were exposed to AdWtp53, and the synthesis of p53 protein assessed by immunoprecipitation. As shown in Figure 2B, MCF-10, MCF-7, Adr^R MCF-7 and MDA-MB-231 cells expressed low levels of endogenous p53. However, following exposure of the cells to 10 pfu/cell of AdWtp53, a marked increase in the rate of p53 protein synthesis was observed in the infected cells. In contrast, infection with AdControl did not result in any increase in p53 expression above that present in uninfected cells. These results were also confirmed by Western blot analysis (see Example 5) and indicate that AdWtp53 can infect both human mammary and lung cells. Moreover, infection with AdWtp53 resulted in high levels of p53 expression in these cells.

EXAMPLE 13**WAF1/Cip1 Expression/Induction Following AdWtp53 and AdWAF1 Infection.**

AdWtp53-mediated p53 expression and its consequence on WAF1/Cip1 induction were examined in cells

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infected with AdWTp53. In parallel experiments, AdWAF1-mediated WAF1/Cip1 expression was also examined in cells infected with AdWAF1. The cell lines studied included: (1) H-358 lung cancer cells, which are devoid of p53 expression (p53 null) (Takahashi, et al., Science 246:491-494 (1989)); MDA-MB-231 human breast cancer cells which express mutant p53 (Casey, et al., Oncogene 6:1807-1811 (1991)); MCF-7 human breast cancer cells which express wild type p53 (Casey, et al., Oncogene 6:1807-1811 (1991)) and normal mammary epithelial cells (NMECs) which also express endogenous wild type p53 (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). The cells were cultured and maintained as set forth in Example 2.

EXAMPLE 14

Western Blot Analysis.

Cells (1×10^6) were plated in 10 cm tissue culture dishes and incubated with adenoviral vectors for 48 hours as described in Example 2. The cells were then scraped and cell lysates subjected to Western blot analysis as previously described (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). The blots were probed with 3 μ g/ml of Ab-2 and Ab-6 for p53, 3 μ g/ml of EA 10 for WAF1/Cip1, with 3 μ g/ml of Actin (Ab-1) antibody. All antibodies were obtained from Oncogene Science (Uniondale, NY). The blots were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with horse radish peroxidase conjugated to secondary antibody and specific complex detected by the enhanced chemiluminescence technique according to the manufacturer's directions (Amersham, Arlington Heights, IL).

The Western blot analysis demonstrates that the level of p53 increased substantially (at least 10-fold) following AdWTp53 (50 pfu/cell) infection in each cell line (Figure 2, Top Panel); and p53 expression in turn induced high levels of WAF1/Cip1 protein in all the cell lines.

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(Figure 9, Middle Panel). Following infection with AdWAF1 (50 pfu/cell), a high level expression of WAF1/Cip1 protein was also observed. (Figure 9, Middle Panel). More importantly, WAF1/Cip1 expression following AdWAF1 infection was comparable to the levels induced by AdWtp53 (50 pfu/cell) infection. In these experiments, AdControl vector did not increase p53 or WAF1/Cip1 expression; and no change in actin protein level was detected in any of the cells lines following infection with either AdControl, AdWtp53 or AdWAF1 (Figure 9, Lower Panel).

EXAMPLE 15

Cell Growth and Cytotoxicity Assays and Effects of AdWtp53 and AdWAF1 on Cell Growth/Killing.

For cell growth measurements, 5×10^4 cells were plated in each well of 6 well tissue culture dishes. After 24 hours, cells were exposed to adenoviral vectors (10 pfu/cell) and incubations continued at 37°C. The cells were trypsinized on each day and counted using a hemacytometer. Cytotoxicity of adenovirus vectors was also assessed after plating cells in 96 well plates (500 cells/well). The cells were incubated with various doses (0- 10^4 pfu/cell) of adenovirus vectors for 7 days at 37°C. The cells were fixed with trichloroacetic acid and stained with 0.4% (wt/vol) sulforhodamine B (Sigma, St. Louis, MO) essentially as described previously (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). An O.D.₅₆₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and used as a measure of cell number.

The cytotoxic effects of p53 and WAF1/Cip1 overexpression were investigated in cells infected with AdWtp53 and AdWAF1. Infection of H-358 and MDA-MB-231 cells with AdWtp53 completely inhibited cell growth over the seven day period examined (Figures 10A and 10B), and the cell number was reduced by day 7 to levels less than half of that present at time 0. Following AdWtp53 infection, MCF-7 cells

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continued to proliferate, although at a slower rate than the control cells (Figure 10C). The growth inhibitory effect of AdWAF1 was much weaker than AdWTp53 in H-358 and MDA-MB-231 cells, but was comparable to that of AdWTp53 in MCF-7 cells (Figures 10A-10C). In cytotoxicity assays, AdWTp53 was about 30-100 times more toxic than AdWAF1 in H-358 and MDA-MB-231 cells, and nearly equally toxic in MCF-7 and NMECs. Thus, the AdWTp53 infection had a more profound growth inhibitory effect as compared to AdWAF1 on cells that were either deficient in p53 or expressed a mutant endogenous p53. However, the growth inhibitory effects of AdWTp53 and AdWAF1 on cells expressing wild-type endogenous p53 were similar, though much weaker.

EXAMPLE 16

Cell Cycle Analysis; Nucleosomal DNA Fragmentation Analysis and the Effect of AdWTp53 and AdWAF1 on Cell Cycle and Apoptosis.

Cells were plated in 6 well tissue culture dishes (2×10^5 cells/well) and infected with adenoviral vectors (50 pfu/cell) for 48 hours. The cells were harvested by trypsinization and resuspended at a concentration of 2×10^5 cells/ml in medium containing 10% FBS, and stored frozen until analyzed. Samples were stained for DNA cell cycle analysis using the rapid nuclear isolation procedure (Wersto, R. P., and Stetler-Stevenson, M. A., Cytometry (in press, 1995)). DNA content was measured using a FACScan flow cytometer (Becton-Dickinson, Mountain view, CA). Cell cycle analysis of the resulting DNA histograms of cell number versus integrated red fluorescence was performed with Multicycle (Phoneix Flow Systems, Dan Diego, CA) using a zero order polynomial to model the S-phase fraction. Debris, cell aggregates, and Go/1 doublets were removed from the cell cycle analysis by software algorithms (Wersto, R. P., and Stetler-Stevenson, M. A., Cytometry (in press, 1995)).

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Cells (2×10^6) were plated in 15 cm dishes and incubated with adenovirus vectors (50 pfu/cell) for 48 hours. Both adherent and floating cells were collected and pelleted by centrifugation at $1800 \times g$ for 5 min (RT-6000B, Du Pont, Boston). Low molecular weight DNA was prepared by a modified Hirt extraction method described previously (Katayose, et al., Clin. Cancer Res. (Submitted 1995); Rosenfeld, et al., Hum. Gene Ther. 5:331-342 (1994)) and evaluated on 2.5% agarose gel electrophoresis.

To investigate the mechanisms of the differential cytotoxicities mediated by wild-type p53 and WAF1/Cip1 overexpression, the effects of AdWtp53 and AdWAF1 infection on the two major growth regulatory mechanisms, cell cycle and apoptosis, were examined.

DNA cell cycle histograms were used to evaluate the percentage distribution of cells in various cell cycle stages in uninfected, AdControl infected, AdWtp53 infected, and AdWAF1 infected cells, and are shown in Figures 11 and 12. In MDA-MB-231 cells (Figures 11A-D; Figure 12A) no effect on cell cycle distribution was observed following infection with AdControl. However, following infection with AdWtp53, a slight increase in cells in G1 phase (from 55 % to 58 %) but a significant decrease in S phase cells (from 24% to 5 %), and a significant increase in the percentage of G2 + M cells from 21 % to 37 % were observed (Figure 11C; Figure 12A). On the other hand, AdWAF1 infection led to an increase in the cell percentage in G1 phase from 55% to 91%, reduction in percentage of cells in S phase from 24% to 2%, and a decline in G2 + M phase from 21% to 7% (Figure 11D; Figure 12A). Thus, while both AdWtp53 and AdWAF1 infection led to a decline in the S phase cells, AdWtp53 caused an accumulation of cells in G2/M phase and AdWAF1 infection resulted in the cell accumulation in G1 phase. These results indicate that WAF1/Cip1 overexpression arrests the cell cycle at G1/S boundary, while p53-overexpression can cause a cell cycle arrest at G2/M stage.

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AdWtp53 and AdWAF1 produced similar effects in H-358 cells. AdWtp53 produced a reduction in S phase and increase in G2 + M phase; while AdWAF1 produced a reduction in S phase and increase in G1 phase (Figure 12B). In MCF-7 and NMECs, AdWtp53 and AdWAF1 infection resulted in declines in the percentage of cells in S phase in MCF-7 and NMECs. However, no significant change in proportion of cells in G2 + M or G1 was apparent following AdWtp53 or AdWAF1 infection of these cells (Figures 12C and 12D).

The effects of AdWtp53 and AdWAF1 on apoptosis was also examined by assaying the nucleosomal DNA fragmentation and cell cycle analyses of both adherent and floating cells. As shown in Figure 13, 24 hours following exposure of H-358 and MDA-MB-231 cells to 50 pfu/cell of AdWtp53, several lower molecular weight DNA bands (DNA laddering of approximately 145 bp) in the range of 145-1050 bp were observed. These DNA fragments are characteristic of cells undergoing apoptosis (Figure 13B). In contrast, exposure of MDA-MB-231 and H-358 cells to AdWAF1, AdControl or mock infection of these cells produced no detectable DNA laddering (Figures 13A and 13B). Furthermore, following exposure of MCF-7 cells to AdWtp53 or AdWAF1 at 50 pfu/cell, no DNA laddering was observed (Figure 13C). NMECs also did not undergo apoptosis following AdWtp53 or AdWAF1 infection.

Cell cycle analysis of both adherent and floating cells showed the appearance of apoptotic cells following AdWtp53 infection in H-358 and MDA-MB-231 as determined by DNA fragmentation on FACS analysis. However, AdWAF1-infection failed to induce apoptotic cells following infection of H-358 and MDA-MB-231 cells (cell cycle pattern of MDA-MB-231 following infection with AdWtp53 and AdWAF1 is shown in Figures 13D and 13E respectively; the apoptotic cells peak is indicated by an arrow). Cell cycle profiles of MCF-7 and NMECs also failed to detect the presence of dead cells following infection with either of the adenoviral vectors.

These results indicate that tumor cells null for p53

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(H-358) or expressing an endogenous mutant p53 (MDA-MB-231) undergo apoptosis following exposure to AdWtp53; while tumor cells (MCF-7) or normal cells (NMECs) expressing wild-type p53 appeared to be resistant to AdWtp53 induced apoptosis. Moreover, overexpression of WAF1 protein following AdWAF1 infection does not appear to mediate apoptosis even in cells null for p53 or expressing endogenous mutant p53. These results suggest that while WAF1/Cip1 protein overexpression can cause cell cycle arrest, overexpression of this protein is not sufficient to induce apoptosis.

EXAMPLE 17

Cell Cycle Analysis; Nucleosomal DNA Fragmentation Analysis and the Effect of Adp27 and Adp16 on Cell Cycle and Apoptosis.

A. Cell Cycle Analysis.

Cells were plated in 6-well dishes (2×10^5 cells/well) and infected with adenoviral vectors (50pfu/cell) for 48 hours. Cells were harvested by trypsinization and resuspended at a concentration of 2×10^5 cells/ml in 100% FBS and stored frozen until analyzed. Samples were stained for DNA cell cycle analysis using the previously described procedure. DNA content was measured using a FACSan flow cytometer (Becton-Dickenson, Mountain View, CA.). Cell cycle analysis of the resulting DNA histograms of cell number versus integrated red fluorescence was performed with Multicycle (Phoenix Flow Systems, San Diego, CA.) using a zero order polynomial to model the S-phase fraction. Debris, cell aggregates, and G0/G1 doublets were removed from the cell cycle analysis by software algorithms.

B. Nucleosomal DNA Fragmentation (Apoptosis) Analysis.

Cells (2×10^6) were plated in 15 cm dishes and the next day incubated with adenovirus vectors (50 pfu/cell) for 24 hours. Both adherent and floating cells were collected and pelleted by centrifugation at $1800 \times g$ for 5 minutes (RT-6000B, Du Pont, Boston). Low molecular weight DNA was

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prepared by a modified Hirt extraction method described previously, and evaluated on 2.5% agarose gel electrophoresis.

C. Cyclin Kinase Assays.

4 x 10⁶ cells were plated in 15 cm dishes. The next day the cells were infected with recombinant adenoviruses (50 pfu/cell) for 24 hours. The cells were then harvested and lysed in a buffer. For cdk2 kinase activity, lysates were immunoprecipitated by anti-cdc2. For cdc2 kinase, cell lysates were immunoprecipitated. For cdc2-cyclin B-1 dependent kinase, lysates were precipitated with anti-cyclin B1. In brief, cell lysates were incubated with 1 ug primary antibody for 1 hour at 40C. Immune complexes were collected on protein A-Sepharose beads. The beads were washed three times with EBC buffer, three times with kinase reaction buffer (20 mM Tris-HCL pH 7.5, 4 mM MgCl₂). The beads were then resuspended in kinase assay mixture containing 80 uM (³²P-ATP), histone H1 (2 ug) (Gibco-BRL). After incubation at 37°C, the reaction was stopped by the addition of 2X Laemli SDS sample buffer. Proteins were separated on 10% SDS-polyacrylamide gels, and the gels were then dried and autoradiographed.

EXAMPLE 18

Ad.RSV8gal Expression in Breast Tumor and Bone Marrow Cells.

A. Cells and Cell Culture.

Breast cancer cell line MDA-MB-231 (ATCC HTB 26) was cultured in Minimal Essential Medium (Zn++ option) containing 10% Fetal Bovine Serum (FBS) (Gibco BRL, Gaithersburg, MD). 293 cells (ATCC CRL 1573), an adenoviral transformed human kidney cell line, was propagated in improved minimal essential medium (Biofluids, Rockville, MD) containing 10% FBS. Human bone marrow cells were obtained from normal donors. Approximately 10 ml of bone marrow aspirates were collected in a 20 ml syringe containing preservative free heparin (Lymphomed, Deerfield, IL) and

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then diluted in Hanks' Balanced Salt Solution without CaCl₂ or MgCl₂ (HBBS) (Gibco-BRL, Gaithersburg, MD). Cells with a density of ≤ 1.077 g/cm³ were separated on a Ficoll-sodium diatrizoate gradient (LSM, Organon Teknika Corp., Durham, NC), washed three times with HBBS, and suspended in an enriched Iscove's Modified Dulbeccos' Medium (Gibco BRL, Gaithersburg, MD) (Clarke, et al., Nature 362:849-852 (1993)).

B. Adenoviral Vectors

dl312, a replication-deficient mutant of adenovirus, (Lowe, et al., Nature 362:847-849 (1993)) Ad.RSV β gal vector (a recombinant Ad vector expressing β -gal gene (Dulic, et al., Cell 76:1013-1023 (1994))), and AdWTp53, a replication-deficient recombinant adenoviral vector expressing human wild-type p53 were propagated in 293 cells and purified by two rounds of CsCl₂ density centrifugation. Adenovirus titers were determined from viral stocks and stored in Tris-Cl pH 7.5 buffer containing 20% glycerol.

C. Plasmid DNAs

A plasmid expressing the bacterial β -galactosidase gene driven by cytomegalovirus early gene promoter, (CMV β -gal) (Nelson, W. G. and Kastan, M. B., Mol. Cell. Biol. 14:1815-1823 (1994)) was used to measure the transfection efficiency of cells. Adenoviral-mediated enhancement of toxicity of plasmid DNA was determined using plasmid pULI100, containing the Pseudomonas exotoxin catalytic domain (minus secretory signals) driven by a CMV promoter (Michalovitz, D., Halevy, O. and Oren, M., Cell, 62:671-680 (1990)).

D. Ad Infections

Human breast cancer cells were plated at the appropriate density. After 24 hours, the medium was changed to OPTI-MEM (Gibco-BRL, Gaithersburg, MD) and the cells were infected with various multiplicity of infection (moi) of adenovirus. After 2 hours, FBS was added to a final concentration of 10% and the incubation continued at 37°C. For human bone marrow cells and human CD34⁺ cells, freshly

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isolated cells were plated in OPTI-MEM and infected with increasing moi of adenoviral vectors. After 2 hours, FBS was added to the final concentration of 10% and the incubation continued at 37°C. Other experimental details such as the number of cells, moi of adenovirus, and the length of incubations are described below for each experiment.

E. Estimation of Adenovirus Receptor Number

Receptor numbers for adenovirus were determined by scatchard analysis of ^{35}S -dI312 binding to cells as described previously. (See Seth, P., Rosenfeld, M., Higginbotham, J. and Crystal, R. G., J. Virol. 68:933-940 (1994)). In brief, 0.2×10^6 cells were used to bind ^{35}S -dI312 adenovirus (10^4 cpm, $0.1 \mu\text{g}$ adenovirus protein) at 40C for 1 hour in the presence of unlabeled dI312 (0-100 μg). Scatchard plots were drawn as described previously. (See Seth, P., Rosenfeld, M., Higginbotham, J. and Crystal, R. G., J. Virol. 68:933-940 (1994)). Binding assays were conducted in triplicate and the mean taken. Table 2 sets forth the receptor numbers for adenoviruses on breast cancer cells and human bone marrow cells.

D. Ad.RSV β gal Expression.

Breast cancer cells and human bone marrow cells (2×10^4) were plated in 96 well plates and infected with increasing concentrations of Ad.RSV β gal (0.06 -5000 pfu/cell) for 24 hours. Cells were then lysed in 100 μl of 20 mM Tris-HCl, containing 0.1% Triton X 100 and 30 μl aliquots used to determine β -gal activity by a calorimetric assay as described previously. An O.D.₄₂₀ of 1 was defined as 1 unit of β -gal activity.

For detecting β -gal expression in individual cells, cells (2×10^4) were plated in 60 mm dish, and infected with 100 pfu/cell Ad.RSV β -gal. Following a 24 hour infection, breast cancer cells were then fixed in phosphate buffered saline, pH 7.5 (PBS) containing 0.5% formaldehyde (mallinckrodt, Inc, Paris, KY) and 0.2% glutaraldehyde

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(Polysciences, Inc, Warrington, PA) at 4°C for 5 min. After washing with PBS, cells were then incubated in a solution containing 0.5 mg/ml X-gal, 10 mM K₄FeCN₆, 10 mM K₃FeCN₆, 10 mM MgCl₂ for 30 min at 37°C to develop the blue color. For CD34⁺ cells the same protocol was used except cells were centrifuged at 3,000 rpm in an eppendorf centrifuge between washing, fixing and staining steps. Cells were photographed using 2000 x magnification.

TABLE 2

**NUMBER OF ADENOVIRUS RECEPTORS ON
BREAST CANCER CELLS AND HUMAN BONE MARROW CELLS**

CELL LINE**RECEPTOR NUMBER****HIGH AFFINITY****LOW AFFINITY**

(1) MDA-MB-231	6.2 x 10 ³	1.1 x 10 ³
(2) MCF-7	5.3 x 10 ³	7 x 10 ²
(3) MDA-MB-453	7.5 x 10 ³	1.8 x 10 ³
(4) Human Bone Marrow	Undetectable	Undetectable

Cells were exposed to ³⁵S-dI312 adenovirus and the adenovirus receptor number was estimated from the scatchard plots drawn from the binding experiments as described in Example 14.

EXAMPLE 19

AdWtp53-mediated Cytotoxicity Assays and Cytotoxic Effects of AdWtp53 in Breast Tumor and Bone Marrow Cells.

Freshly trypsinized breast cancer cells were mixed with CD34⁺ human bone marrow cells and exposed to different moi of AdWtp53 for 2 hours. Cell cultures were then split; half of the cells (500 cells) were plated in 60 mm dishes and grown in IMEM containing 10 % FBS to form colonies of breast cancer cells. After 14 days the colonies were stained with 5 mM methylene blue, and counted. The other half (500 cells) of the cell suspension was cultured in 0.8 % methyl cellulose medium containing 5 % PHA-LCM (Stem Cell

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Technologies, Vancouver, BC) to determine colony forming units of granulocytes/macrophages. One ml of methyl cellulose medium containing cells was placed in a 35 mm gridded tissue culture dish (Nunc, Naperville, IL), incubated at 37°C, and the CFU-GM colonies counted after 14 days.

Further, the cytotoxic effects of an Ad vector expressing human wild type p53 (AdWTp53) were determined. AdWTp53 was shown to be cytotoxic to breast cancer cells. Following infection of MDA-MB-231 breast cancer cells and CD 34⁺ bone marrow cells with increasing moi of AdWTp53, the cytotoxicity of AdWTp53 was measured by colony forming assays. As shown in Figure 16, following infection of MDA-MB-231 cells with AdWTp53, significant cytotoxicity (about 55% decline in colony numbers) was observed at an moi of 8 pfu/cell. At moi 100 pfu/cell or higher, essentially no colony formation was observed. Bone marrow cells, on the other hand, were much more resistant to killing by AdWTp53. At an moi of up to 1000 pfu/cell, there was essentially no decline in CFU-GM. However, at a very high moi ($\geq 10,000$ pfu/cell) of AdWTp53 there was about 50% reduction in colony numbers (Figure 16). These results indicate that MDA-MB-231 breast cancer cells are at least 3 orders of magnitude more sensitive to the cytotoxic effects of AdWTp53 compared to human CD34⁺ human bone marrow cells.

EXAMPLE 20

Ad and Lipofectamine-mediated Enhancement of CMV β -gal Expression and Ad-mediated Transfection of a CMV β -gal Plasmid in Breast Tumor and Bone Marrow Cells.

Cells (2×10^4) were plated in 96 well plates and exposed to CMV β -gal plasmid (1 μ g) in the absence and presence of dl312 (0.01-100 PFU/cell). To test the effects of lipofectamine, CMV β -gal plasmid DNA (1 μ g) was pre-incubated with different concentrations of lipofectamine (1 μ g) at room temperature for 20 min and used for transfection assays. Following exposure of cells to these reagents for

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2 hours, FBS was added for a final concentration of 10% and the cells incubated at 37°C for 24 hours. Breast tumor cells were then washed twice with PBS and lysed in 20 mM Tris-Cl pH 7.5 containing 0.1% Triton X-100. The same protocol was used for bone marrow cells except the cells were centrifuged at 3,000 rpm, washed twice with PBS and then lysed in the lysis buffer. Aliquots (50 μ l) of cell lysates were used to determine β -gal activity.

Using CMV β -gal, a plasmid expressing the β -gal gene, the adenoviral-mediated enhancement of the plasmid DNA delivery to MDA-MB-231 and bone marrow cells was determined. As shown in Figure 17, MDA-MB-231 cells transfected with plasmid DNA alone, expressed low levels (< 0.01 unit) of β -gal activity. However, in the presence of an increasing concentration of dl312, there was an increase in β -galactosidase activity. The enhancement of the β -gal plasmid expression in this breast cancer cell line was dependent upon the concentration of dl312 used, with maximum β -galactosidase expression (0.65 unit) observed in the presence of 100 pfu/cell. β -galactosidase activity was undetectable in bone marrow cells in the presence of CMV β -gal plasmid alone or in the presence of CMV β -gal plasmid and low concentrations of dl312. Only at a moi of 100 pfu/cell of dl312 was there any detectable β -gal activity (0.007 unit) observed in bone marrow cells (Figure 17). Thus, at an moi of 100 pfu/cell, dl312 infection enhanced the expression of β -gal plasmid DNA in MDA-MB-231 cells 100-fold more than the expression in bone marrow cells.

It is known that adenoviral-mediated increase in plasmid DNA uptake and expression are augmented by the addition of cationic molecules. Therefore, the effect of the polycationic liposome lipofectamine on dl312-mediated CMV β -gal plasmid expression in both human breast cancer cells and human bone marrow cells was determined. As shown in Table 3, dl312 or lipofectamine, when used individually, increased the β -gal activity in MDA-MB-231 cells (0.65 and

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0.58 unit respectively). Furthermore, the presence of both dl312 and lipofectamine increased the β -galactosidase expression in MDA-MB-231 cells 3.8 units (Table 3). Conversely, bone marrow cells showed very little β -galactosidase activity (0.007 and 0.023 units respectively) in the presence of either dl312 (100 pfu/cell) or lipofectamine alone. Even in the presence of both dl312 and lipofectamine, only 0.04 unit of β -gal activity was obtained (Table 3). This level of activity is approximately 100-fold less than that obtained in MDA-MB-231 cells.

Table 3

dl312 and Lipofectamine-Mediated Transfection of CMV β -gal Plasmid in MDA-MB-231 and Human Bone Marrow Cells.

Treatment	β -gal activity (MDA-MB-231 cells)	β -gal activity (Bone marrow cells)
DNA alone	0.001	0.006
DNA + dl312	0.650	0.007
DNA + lipofectamine	0.580	0.023
DNA + dl312 + lipofectamine	3.800	0.040

Cells (2×10^5) were transfected with CMV β -gal plasmid (5 μ g/ml) in the absence and presence of dl312 (10 pfu/cell) and lipofectamine (1 μ g/ μ g DNA) for 24 h. β -gal activity was then estimated as described in Example 14. The values shown are the average of the triplicate determinations.

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EXAMPLE 21

Adenovirus and Lipofectamine-mediated Enhancement of pULI100 Cytotoxicity and Adenoviral-mediated Enhancement of the Cytotoxicity of a Plasmid DNA Expressing Pseudomonas Exotoxin Gene in Breast Tumor and Bone Marrow Cells.

Breast tumor cells were plated in 96 well plates (500 cells/well) and exposed to pULI100 plasmid (1 μ g/well), in the absence or presence of different concentrations of dl312 (0.32-1000 pfu/cell) for 24 hours. To examine the effects of lipofectamine, plasmid DNA was pre-incubated with lipofectamine (1 μ g lipofectamine/1 μ g plasmid DNA). The cells were then exposed to these reagents in OPTI-MEM for 2 hours, after which the serum concentration was raised to 10%. The cells were incubated for 7 days at 37°C. The cells were then fixed in 10% TCA, stained with 0.4% sulforhodamine B (Sigma, St. Louis, MO) and as a measure of cell number, the O.D.₅₆₄ was taken using Bio Kinetic Reader EL 340 (Bio-Tek Instruments). The survival rates of each experimental condition were calculated assuming 100% survival of untreated control cells. The survival rate of CD34+ cells following transfection in the presence of dl312 and pULI100 was estimated by the clonogenic assays described above.

Further, it was determined whether low doses of dl312 could enhance the delivery of a plasmid DNA coding for a toxin gene as measured by the cytotoxicity. To test this, MDA-MB-231 breast tumor cells were transfected with pULI100 DNA, a plasmid containing the cDNA encoding for the catalytic component of Pseudomonas exotoxin which is cytotoxic to cells. Transfections were conducted in the absence and presence of dl312 and/or lipofectamine, and cell survival was examined. As shown in Figure 17, exposure of MDA-MB-231 cells to pULI100 (1 μ g) or a fixed concentration of either dl312 (10 pfu/cell) alone or lipofectamine (1 μ g) alone did not result in any significant cell killing. However, in the presence of either dl312 or lipofectamine,

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pULI100 transfection resulted in > 70 % killing of MDA-MB-231 cells. When pULI100 transfections were performed in the presence of both dl312 and lipofectamine, greater than 95% cell killing was observed (See Figure 18). On the other hand, when human bone marrow cells were exposed to similar concentrations of pULI100 plasmid alone or in combination with dl312 and lipofectamine, less than 5% cells appeared to be killed (See Figure 18). Even when a combination of 100 pfu/cell of AdWtp53, pULI100 plasmid and lipofectamine was used, while CD34+ bone marrow cells were fairly resistant to killing (in colonogenic assays only a decline of about 20 % colony number was obtained), in MDA-MB-231 cells, AdWtp53 alone caused 100% reduction in the colony number. These results indicate that while exposure of breast tumor cells to a low concentration of adenovirus, lipofectamine, and a plasmid coding for a toxin gene are very cytotoxic to breast tumor cells, human bone marrow cells are relatively resistant to these treatments.

EXAMPLE 22

AdCD-Mediated Cytosine Deaminase Assays and Cytotoxic Effects of AdCD in Breast Tumor Cells.

A. Cytotoxicity Assays.

Cytotoxicity of adenovirus vectors was assayed after plating cells in 96 well plates (500 cells/well). Cells were incubated with various doses ($0-10^4$ pfu/cell) of adenovirus vectors in the absence and presence of different concentrations of 5-Fluoro-cytosine (5-FC) for 7 days at 37°C. The cells were fixed with trichloroacetic acid and stained with 0.4% (wt/vol.) sulforhodamine B (Sigma, St. Louis, Mo.) essentially as described. An O.D.₅₆₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and used as a measure of cell number. In the absence of 5-FC, there was no detectable basal activity of CD in either mock infected or AdControl infected cells (Table 4). However, following infection of cancer cells

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with AdCD, very high CD activities were observed. CD expression was dependent upon the dose of AdCD used (See Table 4). In the presence of different concentrations of 5-FC, up to 100 pfu/cells AdCD had no cytotoxicity in either MDA-MB-231 or MCF-7 cells. Similarly, when cells were exposed to different concentrations of 5-FC alone, no significant toxicity was observed. However, when the cytotoxicity assays were performed in the presence of increasing concentrations of AdCD and 5-FC, significant increases (up to 2-3 log) increases in the cytotoxicities of AdCD and 5-FC were detected. These results indicated that the combination of AdCD and 5-FC was cytotoxic to breast cancer cells. However, when cytotoxicity assays were conducted in the presence of AdControl, no significant enhancement of 5-FC cytotoxicity was observed indicating the specificity of AdCD.

Table 4
AdCD-Mediated CD Activity in Breast Cancer Cells

Cell line	CD activity				
	UI	AdControl (10 pfu/cell)	AdCD (10 pfu/cell)	AdControl (100 pfu/cell)	AdCD (100 pfu/cell)
MDA-MB-231	0	0	53170	0	584347
MCF-7	0	0	83246	0	1117224

B. Bystander Effects of AdDC.

To determine if bystander effects contributed to the overall toxicity of AdCD, MDA-MB-231 cells were infected with AdCD (10 pfu/cell) and mixed in different ratios with mock infected cells. Briefly, cells were plated in 10 cm dishes (1×10^6 cells/dish). 24 hours later the cells were infected with either AdControl (10 pfu/cell) or AdCD (10

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pfu/cell). The next day, AdCD-infected cells were mixed with uninfected cells in varying ratios (0.0001 to 100%) and plated in 96-well dishes. A total 20,000 cells were plated in each well. Immediately after plating the cells, 5-FC was added (1-5 mM) and the cells were incubated at 37°C for 5 days. The cell viability was estimated using a MTT assay as described previously. In the presence of 5 mM 5-FC alone, the uninfected cells were 100% viable. However, when the cells were incubated with 5-FC, and either 100, 50, 20 or 10% of the infected cells; the viability of the total cell population was reduced to 0%. However, when the proportion of infected cells was less than 10%, some viable cells were still present at the end of the 5-day period. From these results it appears that to kill 100% of the population, only about 10% of the cells need to be infected by AdCD, which is likely to be due to bystander effects of the AdCD in the presence of 5-FC.

C. Biochemical Measurement of 5-FC Conversion to 5-FU.

Breast cancer cells were plated in 10 cm dishes (2 x 10⁶ cells/dish). The next day the cells were infected with either AdCD or AdControl for 24 hours. Cells were then washed with PBS, scraped and suspended in buffer. Cytosine deaminase activity was then measured.

EXAMPLE 23

Effect of AdCD Infection in Human Xenografts in Nude Mice.

In vivo effects of AdCD were examined in human breast tumors MDA-MB-231 grown as xenografts in nude mice. MDA-MB-231 cells (5 x 10⁶) were injected in nude mice subcutaneously. After 2 weeks, palpable tumors were visible. A single injection of AdCD or AdControl (10⁹ pfus in 0.1 ml) was given intratumorally. Immediately after this, the animals were administered with 5-FC (5 mg/kg body weight). 5-FC was administered twice each day for 5 days. Tumor sizes were measured each week. As shown in Figure 20, the tumors of animals which received AdControl or AdCD alone

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continued to grow until day 21. However, further development of tumors of animals which received AdCD in conjunction with 5-FC was significantly inhibited. In contrast, there was no significant effect on the further growth of the tumors of animals which received AdCD and 5-FC.

EXAMPLE 24

Effect of AdWtp53 Infection in Human Xenografts in Nud Mice.

A. Apoptosis Assays.

The effects of p53 overexpression on apoptosis were investigated in MDA-MB-231 human breast cancer cells using terminal deoxyribonucleotidyl transferase immunostaining. MDA-MB-231 cells were plated in 10 cm dishes (2×10^6 /dish) and 24 hour later, medium was changed to IMEM containing 2% FBS. Cells were infected with either AdControl or AdWtp53 (50 pfu/cell). After 2 hours, the serum concentration was raised to 10% and incubation continued at 37°C for another 24 hours. Cells were harvested, fixed in 70% ethanol, and apoptotic cells detected via a terminal transferase reaction using Apotag kit (Oncor, Gaithersburg, Maryland). Cells were photographed using a fluorescent microscope (1000x magnification). As shown in Figure 19, following infection with AdControl, only a very few bright fluorescent cells (indicative of apoptosis) were visible (left panel). However, following infection with AdWtp53 there were large numbers of brightly fluorescent cells (right panel) indicating the induction of an apoptotic pathway by wild type p53 transgene expression.

B. Nude Mice Studies.

In vivo effects of AdWtp53 were examined in human breast tumors MDA-MB-231 grown as xenografts in nude mice. 2 week old athymic mice (nu/nu) (Frederick Cancer Research Facility, Frederick, Maryland) were used in this study as an animal model for tumor growth. MDA-MB-231 cells were grown

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in monolayers. Prior to injections, cells were trypsinized, washed, and suspended in phosphate buffered saline. The mice were injected with 10^7 MDA-MB-231 cells in 0.1 PBS subcutaneously (5×10^6 cell/site) using a 21 g needle. Tumors were allowed to develop for 14 days. On day 14, tumors were either injected with AdWtp53 or AdControl (10^9 pfu/tumor) in the middle of the tumor using a 25G needle. An additional injection of either AdWtp53 or AdControl was administered on day 21 and the animals photographed on day 28. (See Figures 21A and 21B).

The results of this study indicate that those tumors of nude mice that were injected with AdWtp53 disappeared completely over the course of the treatment (See Figure 21A), while the tumors of the nude mice that were injected with AdControl (adenovirus only) increased in size to a final volume of 913 mm^3 in 21 days (See Figure 21B). Therefore, adenoviral vectors are useful for the eradication of cancer cells by contacting the cancer cells of the tumor with an amount of the adenoviral vector sufficient for the eradication of the cancer cells.

The results of this study also indicate that adenoviral vectors are useful in the prevention of the development of cancer cells in those subjects who are at risk of developing cancer. The preventative treatment involves the administration of an adenoviral vector expressing the desired DNA to a subject in an amount effective to prevent the development of cancerous cells.

EXAMPLE 25

Effect of AdWtp53 on Adriamycin and Mitoxantrone Resistant Human Breast Cancers.

To demonstrate that recombinant adenoviruses can be used to treat drug resistant cancers, cytotoxicity of AdWtp53 was studied in two human breast cancer MCF-7 cell lines which are resistant to adriamycin (MCF-Adr) and mitoxantrone (MCF-Mito).

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A. Cytotoxicity assays.

Cytotoxicity of adenovirus vectors was assessed after plating cells in 96 well plates (500 cells/well). The cells were incubated with various doses ($0-10^4$ pfu/cell) of adenovirus vectors for 7 days at 37°C. The cells were fixed with trichloroacetic acid and stained with 0.4% (wt/vol) sulforhodamine B (Sigma, St. Louis, MO) essentially as described previously (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). An O.D.₅₄₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and used as a measure of cell number.

B. Western blot analysis.

Cells (4×10^6) were plated in 15 cm tissue culture dishes and incubated with adenoviral vectors for 24 hours as described in Example 9. The cells were then scraped and cell lysates subjected to Western blot analysis as previously described (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). The blots were probed with 3 μ g/ml of Ab-2 and Ab-6 for p53, 3 μ g/ml of EA 10 for WAF1/Cip1, with 3 μ g/ml of Actin (Ab-1) antibody. All antibodies were obtained from Oncogene Science (Uniondale, NY). The blots were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with horse radish peroxidase conjugated to secondary antibody and specific complex detected by the enhanced chemiluminescence technique according to the manufacturer's directions (Amersham, Arlington Heights, IL).

C. Cell Cycle Analysis.

Cells were plated in 6-well dishes (2×10^5 cells/well) and infected with adenoviral vectors (50 pfu/cell) for 48 hours. Cells were harvested by trypsinization and resuspended at a concentration of 2×10^5 cells/ml in 100% FBS and stored frozen until analyzed. Samples were stained for DNA cell cycle analysis using the previously described procedure. DNA content was measured using a FACSscan flow cytometer (Becton-Dickenson, Mountain View, CA.). Cell cycle analysis of the resulting DNA histograms of cell

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number versus integrated red fluorescence was performed with Multicycle (Phoenix Flow Systems, San Diego, CA.) using a zero order polynomial to model the S-phase fraction. Debris, cell aggregates, and G0/G1 doublets were removed from the cell cycle analysis by software algorithms.

D. Nucleosomal DNA Fragmentation (Apoptosis) Analysis.

Cells (2×10^6) were plated in 15 cm dishes and the next day incubated with adenovirus vectors (50 pfu/cell) for 24 hours. Both adherent and floating cells were collected and pelleted by centrifugation at $1800 \times g$ for 5 minutes (RT-6000B, Du Pont, Boston). Low molecular weight DNA was prepared by a modified Hirt extraction method described previously, and evaluated on 2.5% agarose gel electrophoresis.

E. Cyclin Kinase Assays.

4×10^6 cells were plated in 15 cm dishes. The next day the cells were infected with different doses (1-200 pfu/cell) of recombinant adenoviruses for 24 hours. The cells were then harvested and lysed in a buffer. For cdk2 kinase activity, lysates were immunoprecipitated by anti-cdc2. For cdc2 kinase, cell lysates were immunoprecipitated. For cdc2-cyclin B-1 dependent kinase, lysates were precipitated with anti-cyclin B1. In brief, cell lysates were incubated with 1 ug primary antibody for 1 hour at 4°C . Immune complexes were collected on protein A-Sepharose beads. The beads were washed three times with EBC buffer and three times with kinase reaction buffer (20 mM Tris-HCL pH 7.5, 4 mM MgCl_2). The beads were then resuspended in kinase assay mixture containing 80 uM (^{32}P -ATP), histone H1 (2 ug) (Gibco-BRL). After incubation at 37°C , the reaction was stopped by the addition of 2X Laemli SDS sample buffer. Proteins were separated on 10% SDS-polyacrylamide gels, and the gels were then dried and autoradiographed.

The IC_{50} value for AdWtp53 in MCF-7 cells was 42 pfu/cell (See Table 5). However, in MCF-Adr and MCF-Mito

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cells, IC_{50} values were much lower (0.128 pfu and 2.4 pfu/cell, respectively.) These results suggest that MCF-Adr and MCF-Mito cells were much more sensitive to the cytotoxic effects of AdWtp53.

Table 5
AdWtp53 Cytotoxicity in Drug Resistant MCF-7 Cells

Cell line	p53 Status	AdWtp53 IC_{50} (pfu/cell)
MCF-7	Wild Type	42
MCF-Adr	Mutant	0.128
MCF-MITOXR	Wild Type	2.4

Western blot analysis of MCF-7, MCF-Adr and MCF-Mito following infection with various doses of AdWtp53 (1, 10 and 100 pfu/cell) showed high levels of p53 protein expression. In each cell line p53-expression was comparable and was dependent upon the dose of AdWtp53 used. However, a control adenovirus did not increase the p53 expression above the basal level. These results indicate that the increased sensitivity of drug resistant cells towards AdWtp53 was not due to higher transgene expression, but perhaps because of a distinct p53-mediated cascade in drug resistant cells.

F. AdWtp53-Mediated Cell Cycle Arrest.

To evaluate the mechanisms of AdWtp53-mediated cytotoxicity, the effects of AdWtp53 on cell cycle and apoptosis were examined. As shown in Figure 24, each cell line has a certain percent distribution of cells in G1, S and G2/M phase (Panel E,F,G). Following infection of cells with increasing doses of AdWtp53 (1-100 pfu/cell) for 24 hours, a dose-dependent reduction in cell population in S phase (shown as hatched curve) was observed in parental MCF-7 (Panel A-C) as well as the MCF-Adr (Panel D-F) and MCF-Mito (Panel G-I) cell lines. In addition to a decline in S-phase cells, in each cell line, accumulation of cells was observed in G1 phase (Figure 37) indicating a p53 induced

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G1/S arrest. In these studies a control adenovirus did not have any significant effect on the cell cycle pattern in any of the cell lines.

Although AdWtp53-mediated growth arrest was observed in all cell lines, there were some striking differences between MCF-7 and the drug resistant cells. In MCF-ADR and MCF-Mito cells, following infection with AdWtp53, there appeared to be a population of cells in sub G0 phase where apoptotic cells should accumulate. This accumulation of apoptotic cells in MCF-Adr and MCF-Mito increased with an increase in the dose of AdWtp53 used.

G. Effects of p53 Overexpression on cdk2 and cdc2-Cyclin B1 kinase.

Since the cell cycle arrest is likely to be related to the cyclin kinase activities, the effects of p53 overexpression on two cyclin kinases was examined. All the cell lines showed a basal level of cdk2 kinase. Following infection with AdWtp53 (1 pfu/cell) had little effect on the kinase activity, however, when a dose of 10 pfu/cell or greater was used, cdk2 kinase activity was significantly inhibited in all the cells examined. However, infection by a control adenovirus, cdk2 kinase activity was not lowered even at higher doses.

cdc2 kinase (cyclin B1 associated) was also expressed in all cell lines at a certain basal level. Following infection of these cells with a control adenovirus, the basal level of the enzyme activity was not reduced. In parental MCF-7 cells, cdc2 kinase was not much affected following AdWtp53 infection. However, infection of MCF-Adr with AdWtp53 resulted in complete inhibition of cdc2 cyclin B1-associated activity. Similar results were obtained with MCF-Mito.

H. AdWtp53-Mediated Apoptosis.

The effect of AdWtp53 infection on apoptosis was further examined in MCF-7 and the drug resistant cells by evaluating the nucleosomal DNA degradation, as described in Example 17.

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While in MCF-7 cells, AdWtp53 infection (100 pfu/cell) failed to induce any nucleosomal DNA degradation, both the drug resistant cells showed DNA laddering following 24 hour infection with AdWtp53. Even increasing the concentration of AdWtp53 up to 500 pfu/cell or increasing the length of incubation with AdWtp53 up to 48 hours did not induce any specific DNA laddering in the parental MCF-7 cells. Control adenovirus (100 pfu/cell) did not induce apoptosis in any of these cells. These results indicate that induction of apoptotic pathway may play an important role in determining the overall cytotoxicity of AdWtp53.

EXAMPLE 25

Consequence of p53 Gene Expression by Adenovirus Vector on Cell Cycle Arrest and Apoptosis in Human Aortic Vascular Smooth Muscle Cells.

A. Cytotoxicity Assays.

Cytotoxicity of adenoviral vectors was assessed in 96 well plates as described previously (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897). Briefly, cells (250 cells/well) were incubated with various doses (0-14⁴ pfu/cell) of adenovirus vectors, and cell number assessed after 7 days.

The amount of virus required to kill 50% cells (IC₅₀) of AdWtp53 was 4 pfu/cell, whereas those of AdWAF1 and AdControl were 800 pfu/cell and 1200 pfu/cell, respectively. These results indicate that AdWtp53 was 200 and 300 times more toxic to AoVSMC than AdWAF1 and AdControl, respectively. This data is compatible with earlier results showing the existence of G1 subgroup in AoVSMC infected with AdWtp53. To determine whether AdWtp53-mediated cell death of AoVSCM occurred by apoptosis, DNA fragmentation analysis was performed. DNA fragmentation in AoVSMC infected with AdWtp53 was not detected. One possible explanation for this is that the number of cells susceptible to apoptosis might be too small in the beginning to detect DNA fragmentation

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because most AoVSMC in this experiment stayed in G1 phase. Another possibility is that the cytotoxicity of p53 to AoVSMC was induced by atypical apoptosis without DNA fragmentation (Oberhammer, F., et al. (1993) EMBO J. 12:3679-3684).

B. Western Blot Analysis.

Cells (1 x 10⁶) were incubated with adenovirus vector for 48 hours. Cell lysates were subjected to Western blot analysis (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897). Blots were probed with 3 ug/ml of antibodies against p53 (Ab-2), p21 (EA1) and actin (AB-1) (Oncogene Science, Uniondale, NJ.) and analyzed using enhanced chemiluminescence technique (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897).

AoVSMC infected with AdWtp53 (50 pfu/cell) showed high levels of p53 expression, whereas AoVSMC infected with AdWAF1, AdControl (50 pfu/cell) or uninfected cells showed the low base level. Importantly, AdWtp53 induced p21 expression probably by direct transcriptional transactivation of the p21 gene. AoVSMC infected with AdWAF1 of 50 pfu/cell had much higher levels of p21 expressed compared to cells infected with AdControl or uninfected cells. This level of p21 expression is similar to p21 expressed by AdWtp53. AdControl slightly increased p21 protein levels as compared to uninfected cells. The protein levels of whole actin were unchanged in AoVSMC infected by AdWtp53, AdWAF1, AdControl, or uninfected cells.

C. Cell Cycle Analysis.

Cell cycle analysis was performed as described previously (Katayose, D. et al, in press, Cell Growth and Differ. (1995). Briefly, 2 x 10⁵ cells were infected with adenoviral vectors (50 pfu/cell) for 48 hours. DNA content was measured using a FACSan flow cytometer (Becton-Dickenson, Mountain View, CA.) Cell cycle analysis of the resulting DNA histograms of cell number versus integrated red fluorescence was performed with multi cycle (Phoenix

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Flow Systems, San Diego, CA) using a zero order polynomial to model the S-phase fraction.

AdControl had no effect on the cell cycle distribution compared to uninfected cells. By contrast, infection with either AdWTp53 or AdWAF1 (50 pfu/cell) decreased the number of S phase cells in dose dependent manner. While both AdWTp53 and AdWAF1 infection decreased S-phase cells, differential effects by AdWTp53 and AdWAF1 were observed; AdWTp53 induced the accumulation of G2/M phase cells (Figure 37, D, G), while AdWAF1 resulted in an increased number of G1 phase cells (Figure 37, C, E). From these results, it can be determined that p53 overexpression induced both G1 and G2/M cell cycle arrest, whereas p21 overexpression arrested cells at G1/S boundary. Additionally, AdWTp53-infected cells (50 pfu/cell) showed the population in G1 subgroup, indicating that some population of cells infected with AdWTp53 underwent apoptosis. No G1 subgroup was observed in cells infected with either AdWAF1 or AdControl.

D. Nucleosomal DNA Fragmentation Analysis.

Cells (2×10^6) was infected with adenoviral vectors (50 pfu/cell) for 48 hours. Both adherent and floating cells were collected and pelleted by centrifugation at 1800 x g for 5 minutes (RT-6000B, Du Pont, Boston). Low molecular weight DNA was prepared and evaluated on 2.5% agarose gel electrophoresis (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897).

EXAMPLE 26

Induction of Cell Cycle Arrest by Adp27 in Human Breast Cancer Cells at G1/S and G2/M Checkpoints.

A. Adp27-mediated p27 expression. AdWTp53-mediated p27 expression was examined in MDA-MG-231 and MCF-7 human breast cancer cells following infection with Adp27. Western blot analyses demonstrated that the level of p27 increased substantially (at least 20-fold) following Adp27 (100 pfu/cell) infection in each cell line (Figure 27, Top

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Panel). In these experiments, AdControl vector did not increase p27 expression; and no change in actin protein level was detected in any of the cells lines following infection with either AdControl or Adp27.

B. Effect of Adp27 on cell cycle arrest. The effects of Adp27 infection on the two major growth regulatory mechanisms, cell cycle and apoptosis was investigated.

The effects of p27 overexpression on DNA cell cycle histograms were studied using different doses of Adp27 (1, 10, 50, 200 pfu/cell) and the results obtained are shown in Table 6. Mock infected cells had a basal distribution of cells (G1--%, S %, G2.M %). At an moi of 1 pfu/cell, there appeared to be an increase in the percentage of cells in G1 phase to %, and a decrease in S phase cells to %, indicating p27-induced G1/S arrest described above. However, at higher concentrations of Adp27 (100 moi or greater), cell number in G1 appeared to decrease with a concomitant increase in the cell number in G2/M stage. Adp27 produced a similar dose-dependent effects on cell cycle in MCF-7 cells. These results indicate that lower amounts of p27 can induce a strong G1/S arrest in breast cancer cells while higher amounts of p27 can either reverse this arrest or has additional check points at G2.M.

C. Effect of p27 Adp27 infection on cdk2 kinase and cdc2 (cyclin B1 associated kinase) activity.

To further investigate the biochemical mechanism of p27-mediated cell cycle control, the effects of Adp27 infection were also examined on the two key kinase activities responsible for G1/S and G2/M transition, which are cdk2 kinase and cdc2 (cyclin B-1 dependent) kinase. MDA-MB-231 cells had a certain basal level of both cdk2 and cdc2(cyclinB1-associated). Following infection of cells with Adp27, a dose dependent inhibition of both the kinase activities was observed. However, a control adenovirus did not cause the inhibition of these enzyme activities; in fact a slight increase in the activities was observed.

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D. Effect of Adp27 on apoptosis.

To investigate if p27-mediated effects on cell cycle progression will induce the cells to undergo apoptosis, the effects of Adp27 infection on the nucleosomal DNA degradation were studied. Interestingly, even a high dose of Adp27 (200 pfu/cell) did not induce any detectable DNA laddering in MDA-MB-231 cells. However, under the same conditions, AdWTp53 infection (100 pfu/cell) led to a DNA laddering typically seen in apoptotic cells. These results therefore indicate that while p27 can inhibit the cell cycle progression at G1/S and G2/M stages, it fails to induce apoptosis.

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- 30
- 35

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANTS: PREM K. SETH AND KENNETH COWAN
- 5 (ii) TITLE OF INVENTION: METHODS OF PREPARATION
AND USE OF ADENOVIRAL VECTORS
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: MORGAN & FINNEGAN, L.L.P.
(B) STREET: 345 PARK AVENUE
(C) CITY: NEW YORK
(D) STATE: NEW YORK
(E) COUNTRY: USA
(F) ZIP: 10154
- (v) COMPUTER READABLE FORM:
- 15 (A) MEDIUM TYPE: FLOPPY DISK
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WORDPERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
- 20 (A) APPLICATION NUMBER: TO BE ASSIGNED
(B) FILING DATE: FEBRUARY 15, 1996
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/390,604
(B) FILING DATE: February 17, 1995
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- 25 (A) NAME: William S. Feiler
(B) REGISTRATION NUMBER: 26,728
(C) REFERENCE/DOCKET NUMBER: 2026-4185PCT
- (ix) TELECOMMUNICATION INFORMATION:
- 30 (A) TELEPHONE: (212) 758-4800
(B) TELEFAX: (212) 751-6849
(C) TELEX: 421792

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- 35 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-92-

- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: No
- (vi) ORIGINAL SOURCE:
- 5 (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:
- 10 (H) CELL LINE:
- (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY:
- (B) CLONE:
- (ix) FEATURE:
- 15 (A) NAME/KEY:
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 20 base pair cDNA
sequence comprising a portion of the
E1 nucleotide sequence.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- 20 TCTTGAGTGCC AGCGAGTAG 20
- (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
- 25 (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: No
- 30 (vi) ORIGINAL SOURCE:
- (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
- 35 (F) TISSUE TYPE:

- 93 -

- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 20 base pair cDNA sequence comprising a portion of the E1 nucleotide sequence.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

10 CAAGGTTTGG CATAGAAACC 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: No

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 18 base pair cDNA sequence comprising a portion of exon seven of the p53 nucleotide sequence.

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTTGGCTCTG ACTGTACC 18

(2) INFORMATION FOR SEQ ID NO:4:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 10 (iii) HYPOTHETICAL: No
- (vi) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
15 (E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:
- (ix) FEATURE:
20 (A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: 21 base pair cDNA
sequence comprising a portion of
exon eight of the p53 nucleotide
sequence.

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTCCGTCCC AGTAGATTAC C 21

(2) INFORMATION FOR SEQ ID NO:5:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 35 (iii) HYPOTHETICAL: No

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- (vi) ORIGINAL SOURCE:
- (A) ORGANISM:
 - (B) STRAIN:
 - (C) INDIVIDUAL ISOLATE:
 - (D) DEVELOPMENTAL STAGE:
 - (E) HAPLOTYPE:
 - (F) TISSUE TYPE:
 - 5 (G) CELL TYPE:
 - (H) CELL LINE:
 - (I) ORGANELLE:
- (ix) FEATURE:
- (A) NAME/KEY:
 - (B) LOCATION:
 - 10 (C) IDENTIFICATION METHOD:
 - (D) OTHER INFORMATION: 21 base pair cDNA
sequence comprising a portion of the
nucleotide sequence of WAF1.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
- AGTCTCAGTT TGTGTGTCTT A 21
- 15
- (2) INFORMATION FOR SEQ ID NO:6:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - 20 (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: No
- (vi) ORIGINAL SOURCE:
- 25 (A) ORGANISM:
 - (B) STRAIN:
 - (C) INDIVIDUAL ISOLATE:
 - (D) DEVELOPMENTAL STAGE:
 - (E) HAPLOTYPE:
 - (F) TISSUE TYPE:
 - (G) CELL TYPE:
 - 30 (H) CELL LINE:
 - (I) ORGANELLE:
- (ix) FEATURE:
- (A) NAME/KEY:
 - (B) LOCATION:
 - (C) IDENTIFICATION METHOD:
 - 35 (D) OTHER INFORMATION: 21 base pair cDNA
sequence comprising a portion of the

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nucleotide sequence of WAF1.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTGCCATCTG TTTACTTCTC A 21

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What is claimed is:

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1. An adenoviral vector construct comprising:
 - a. an origin of replication;
 - b. a left inverted terminal repeat;
 - c. a nucleotide sequence of the adenoviral genome, said sequence containing a first Clal restriction enzyme site and a second Clal site at the 5' end of the adenoviral genome; and
 - d. a homologous recombination domain.
2. The adenoviral vector of claim 1 further comprising DNA encoding suitable regulatory elements so as to effect expression of the polypeptide in a suitable host cell.
3. A circular, closed vector of claim 2.
4. The adenoviral vector of claim 2 further comprising heterologous DNA encoding a protein.
5. The vector of claim 4, wherein said vector is eucaryotic DNA.
6. The vector of claim 4, wherein the polypeptide encoded by the DNA is selected from the group consisting of cytosine deaminase, Jun/Fos dominant negative mutant, NO-synthetase, p27, GADD 45, p16, p15, mdm2, Rb, BAX, IL2, GMCF, p-53 antisense, Her/Neu2 antisense, and Erb4 antisense.

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7. The vector of claim 4 designated AdWTp53 and having ATCC Accession No. 97064.
8. A method of constructing a vector by homologous recombination between a shuttle vector and ClaI cut genomic DNA derived from claim 1.
9. The method of claim 8 wherein the shuttle vector is selected from the group consisting of pDK10, pDK13, pCG1, pPS1 and pCG2.
10. The adenoviral vector produced by the method of claim 8 further comprising DNA encoding suitable regulatory elements so as to effect expression of the polypeptide in a suitable host cell.
11. A circular, closed vector of claim 10.
12. The adenoviral vector of claim 10 further comprising heterologous DNA encoding a protein.
13. The vector of claim 10, wherein said vector is eucaryotic DNA.
14. The vector of claim 10, wherein the polypeptide encoded by the DNA is selected from the group consisting of NO-synthetase, GADD 45, p16, p15, mdm2, Rb, BAX, IL2, GMCF, p-53, p-53 antisense, Her/Neu2 antisense, and Erb4 antisense.

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15. A host cell containing the vector construct of claim 10.
16. The vector of claim 10 designated AdWAF1 and having ATCC Accession No. 97063.
- 5 17. The vector of claim 10 designated Adp27 and having the structure shown in Figure 25.
18. The vector of claim 10 designated AdCD and having the structure shown in Figure 29.
- 10 19. The vector of claim 10 designated Adp16 and having the structure shown in Figure 31.
- 15 20. The vector of claim 10 designated AdTAM67 and having the structure shown in Figure 33.
21. The vector of claim 10 designated AdB7-1 and having the structure shown in Figure 34.
- 20 22. The vector of claim 10 designated AdB7-2 and having the structure shown in Figure 35.
- 25 23. A method for inhibiting the proliferation of cells, comprising contacting the cells with an amount of the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 effective to inhibit cell proliferation.
- 30 24. The method of claim 23 wherein the cells are epithelial cells.

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25. The method of claim 24 wherein the epithelial cells are mammary epithelial cells or lung epithelial cells.
26. The method of claim 23 wherein the cells are cancer cells.
- 5 27. The method of claim 23 wherein the cancer cells are selected from the group consisting of human melanoma cells, human mammary tumor cells, human lung tumor cells, human sarcoma cells or carcinoma cells.
- 10 28. The method of claim 23 wherein the mammary tumor cells are selected from the group consisting of MDA-MB-231, MCF-7, MCF-Adr and MCF-Mito.
- 15 29. The method of claim 23 wherein the lung tumor cells are H-358 cells.
- 20 30. The method of claim 23 wherein the cancer cells are resistant to drugs.
- 25 31. A composition for inhibiting the proliferation of cells comprising the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 and a suitable carrier.
- 30 32. A method of treating a subject suffering from abnormal cell proliferation, comprising administering to the subject an amount of the
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composition of claim 31 effective to inhibit the abnormal cell proliferation.

33. The method of claim 32, further comprising administering the composition in conjunction with a chemotherapeutic agent.

34. The method of claim 32, further comprising administering the composition in conjunction with irradiation treatment.

35. The method of claim 32 wherein the abnormally proliferating cells comprise tumor cells.

36. The method of claim 35 wherein the tumor cells are selected from the group consisting of human melanoma cells, human mammary tumor cells or human sarcoma cells.

37. The method of claim 32 wherein the abnormal cell proliferation comprises abnormal vascularization.

38. The method of claim 32, wherein the mode of administration of the composition is selected from the group consisting of intravenous, subcutaneous, intramuscular, intratumor or local.

39. The method of claim 32, wherein the mode of administration of the composition effective to inhibit the abnormal cell proliferation is between about 10^6 and 10^8 plaque forming units per tumor.

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40. A method of treating a subject suffering from a tumor which comprises administering to the subject an effective amount of the composition of claim 31 effective to eradicate the tumor cells.
- 5 41. The method of claim 40, wherein the tumor cells are selected from the group consisting of human melanoma cells, human mammary tumor cells, human lung tumor cells, human sarcoma cells or carcinoma cells.
- 10 42. A method of treating a subject at risk of developing cancer which comprises administering to the subject an effective amount of the composition of claim 31 effective to prevent the development of cancerous cells.
- 15 43. A pharmaceutical composition comprising the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 in an amount effective to inhibit cell proliferation, and a pharmaceutically acceptable carrier.
- 20 44. A pharmaceutical composition for purging bone marrow cells or contaminating tumor or cancer cells comprising the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 in a therapeutically effective amount and a
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pharmaceutically acceptable carrier.

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45. A method of purging bone marrow cells of
contaminating cancer cells ex vivo by
contacting the contaminating cells with an
amount of the vector construct of claim 4, or
5 claim 7, or claim 12, or claim 16, or claim
17, or claim 18, or claim 19, or claim 20, or
claim 21, or claim 22 to effectively
eradicate the contaminating cells.

10 46. The method of claim 45 further comprising re-
introducing the purged bone marrow cells into
a patient.

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FIGURE 1

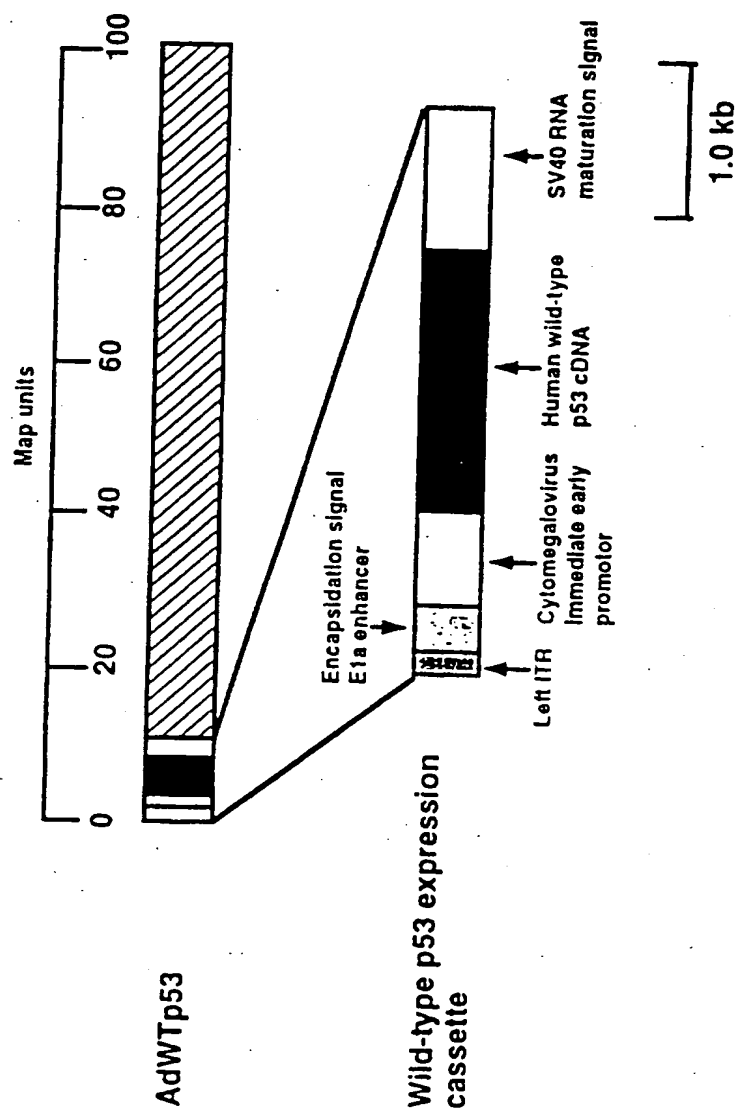
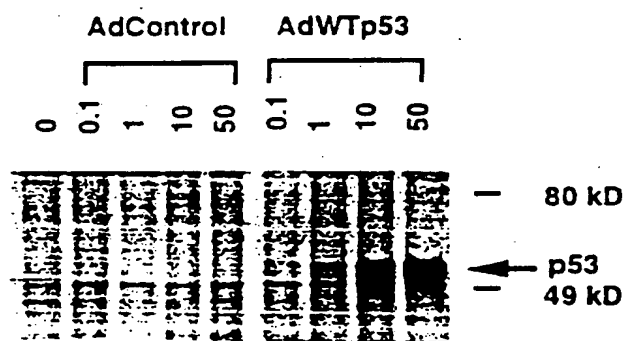
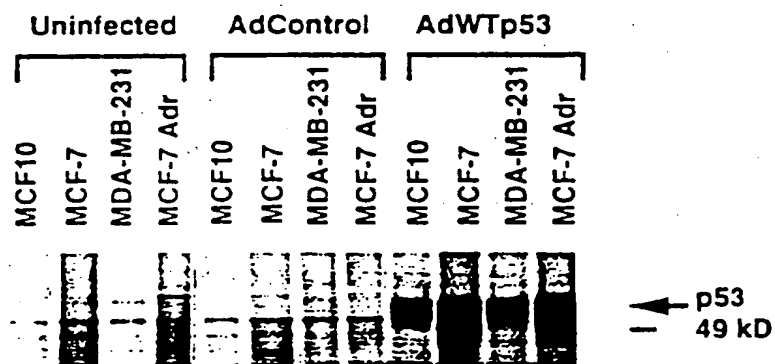
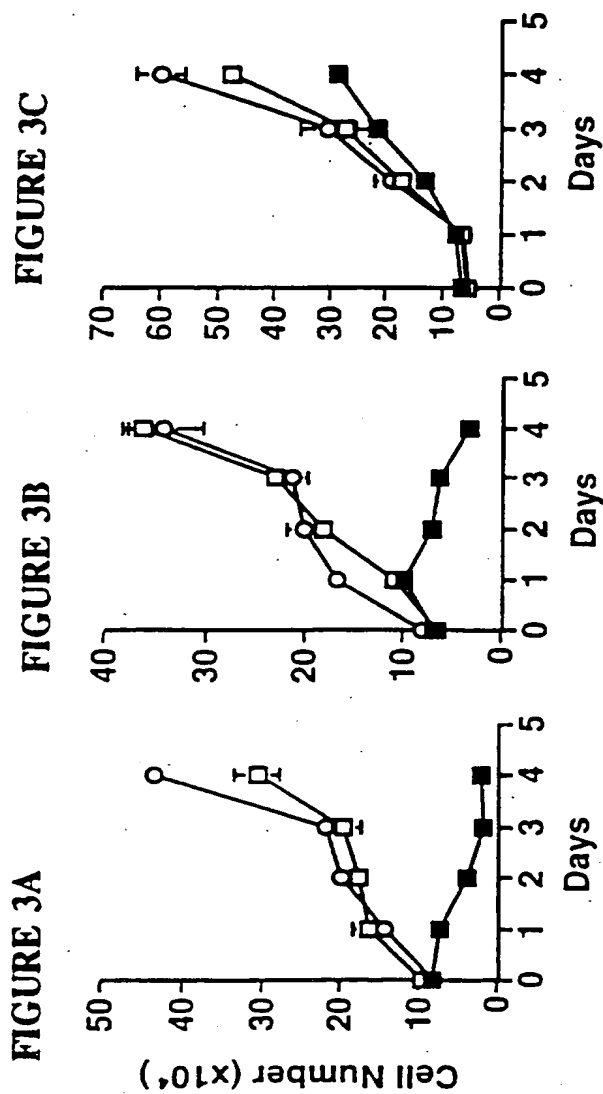
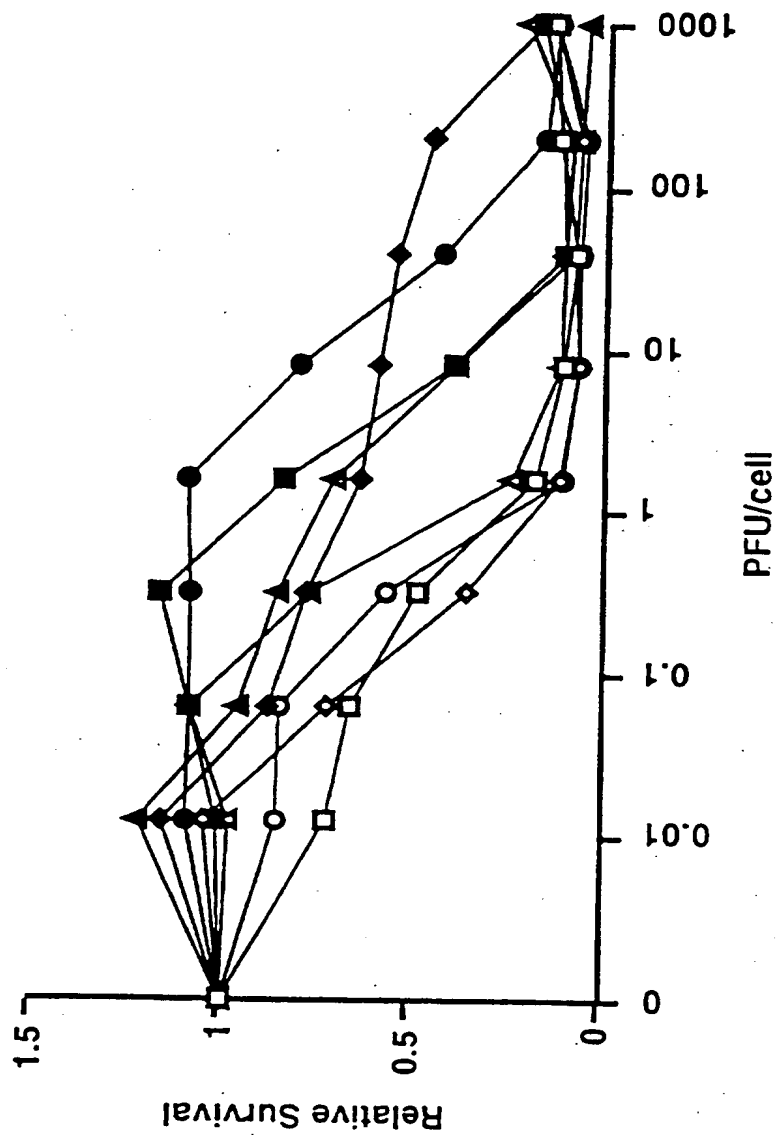


FIGURE 2A**FIGURE 2B**



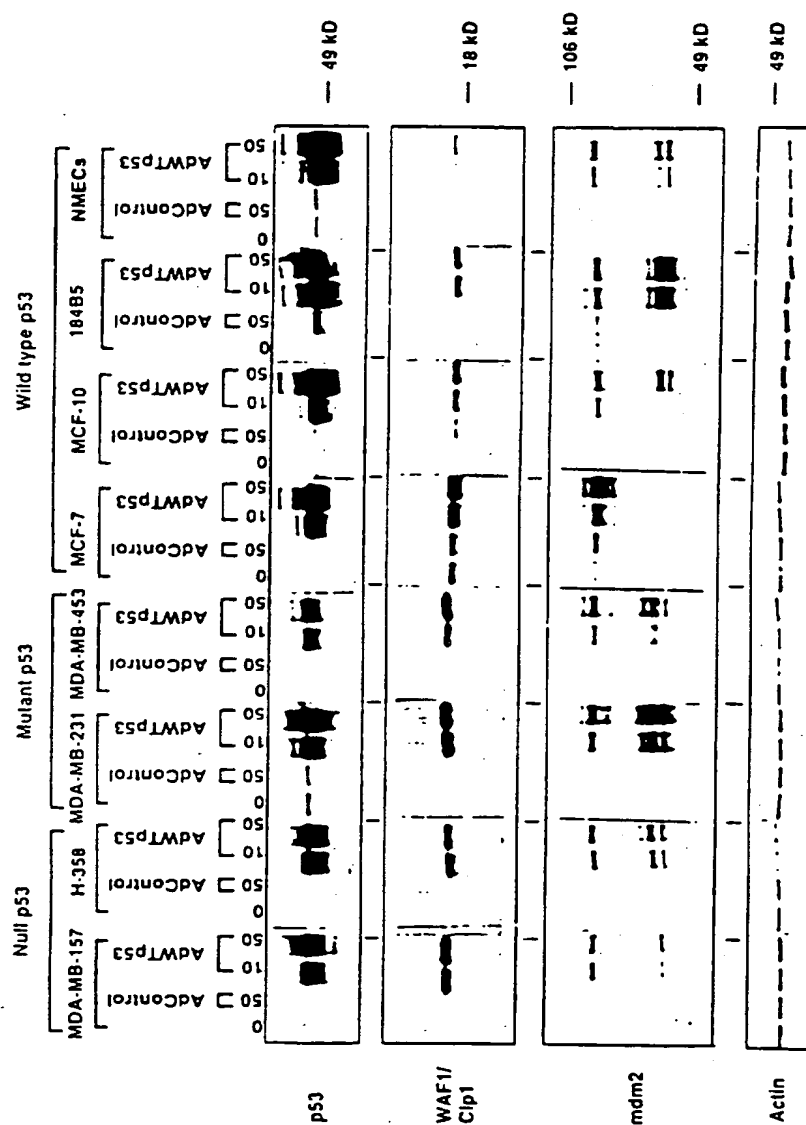
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FIGURE 4



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FIGURE 5



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FIGURE 6

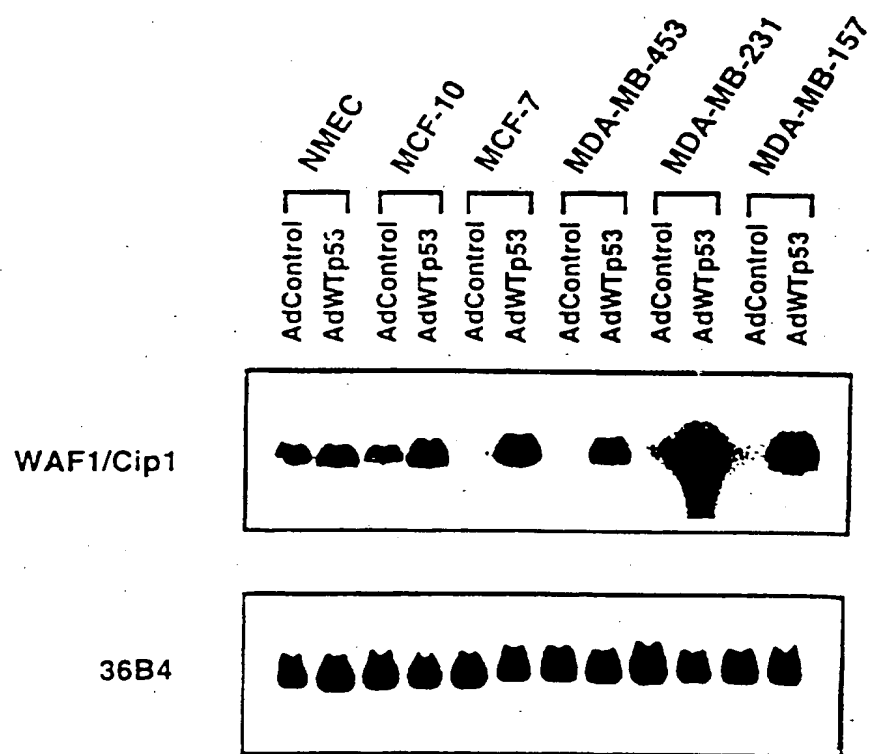
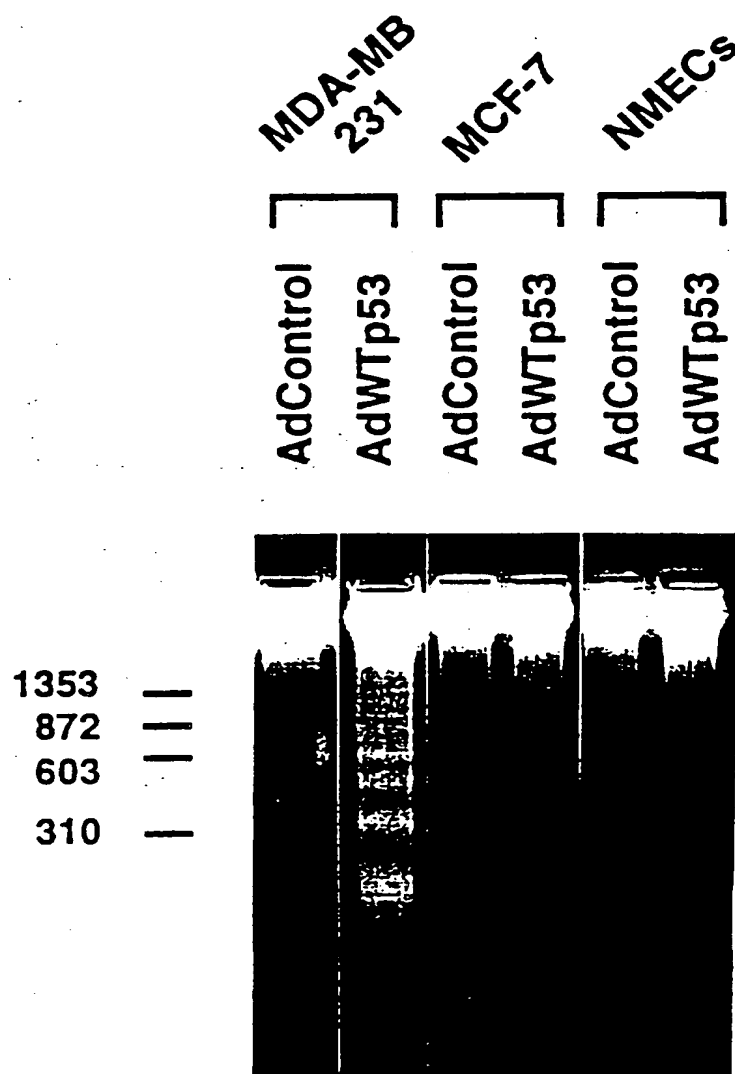


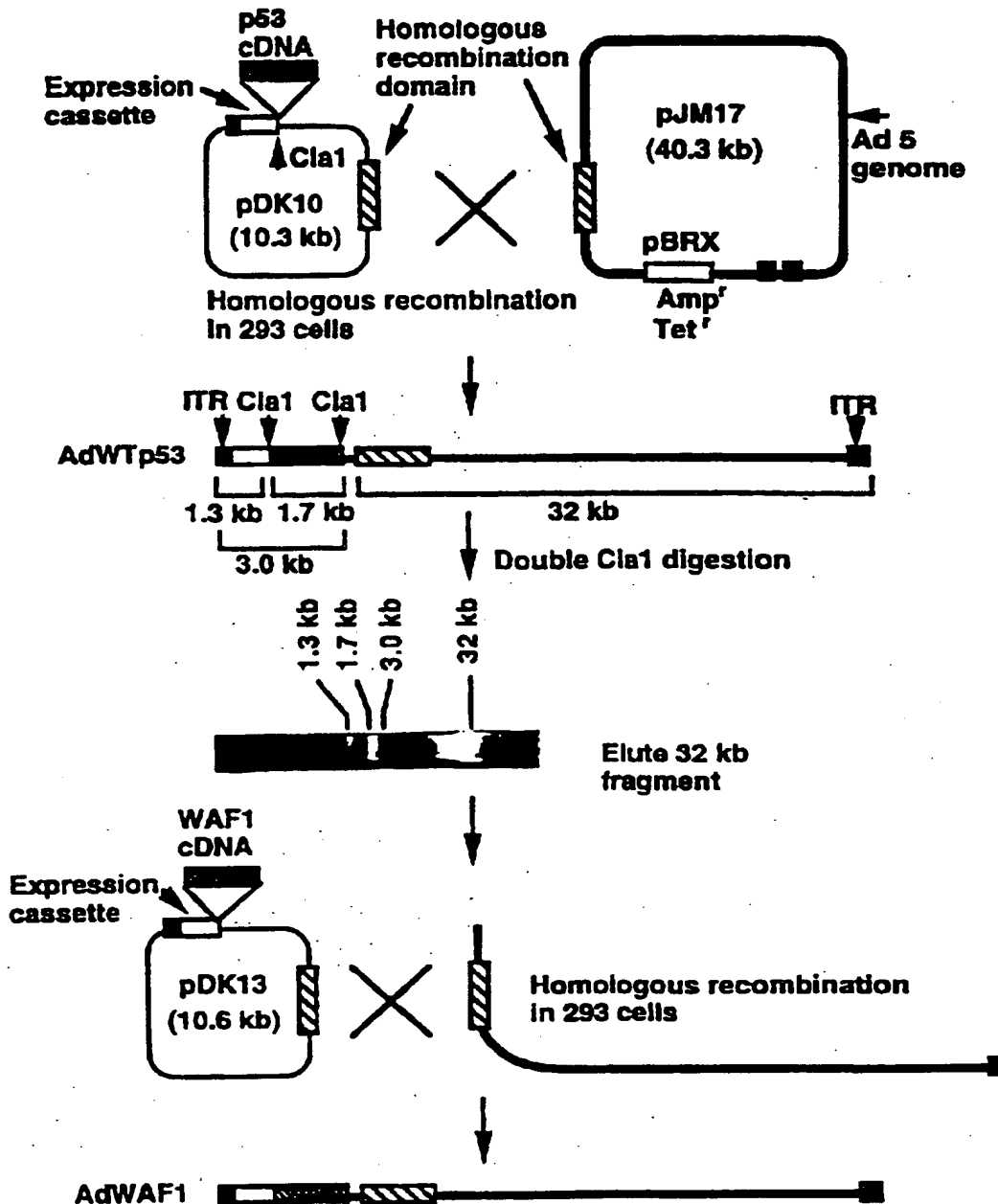
FIGURE 7



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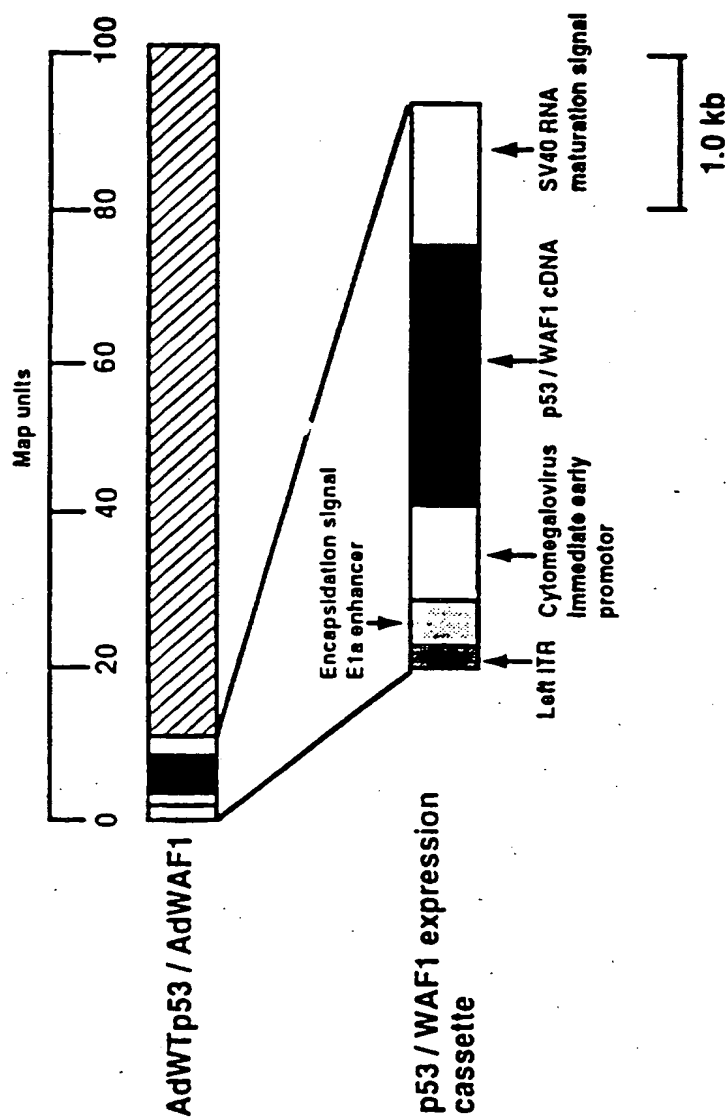
FIGURE 8

The Construction of AdWtp53 and AdWAF1



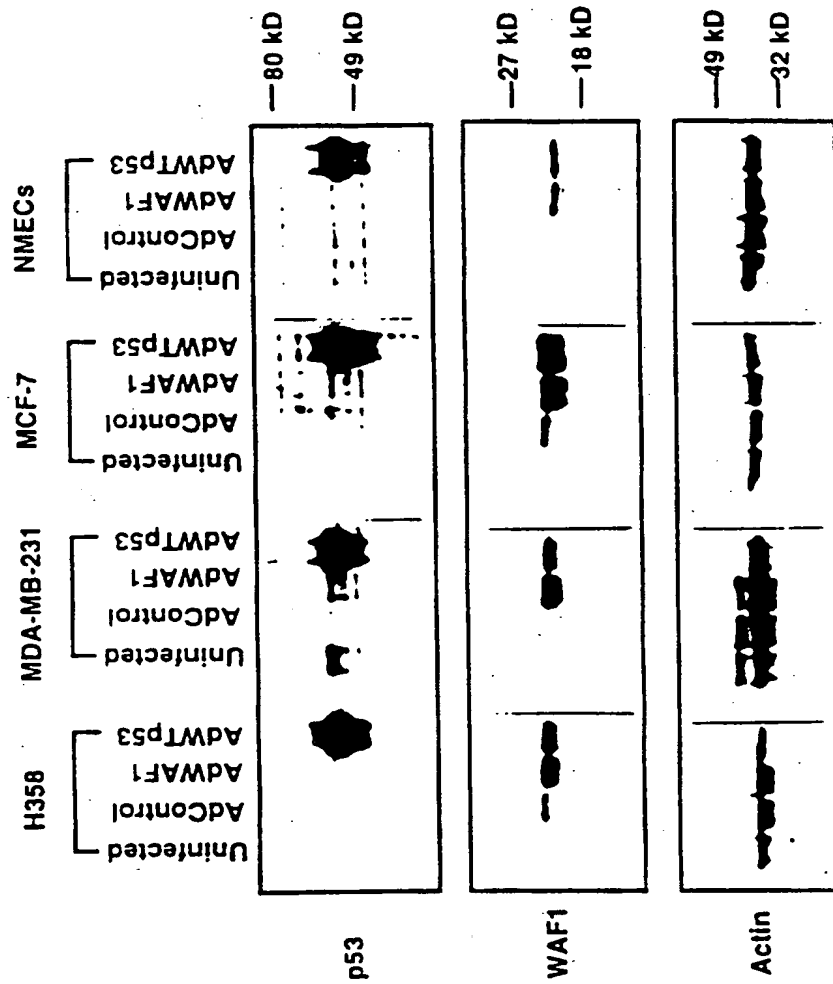
9 / 4 1

FIGURE 9



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FIGURE 10



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FIGURE 11C

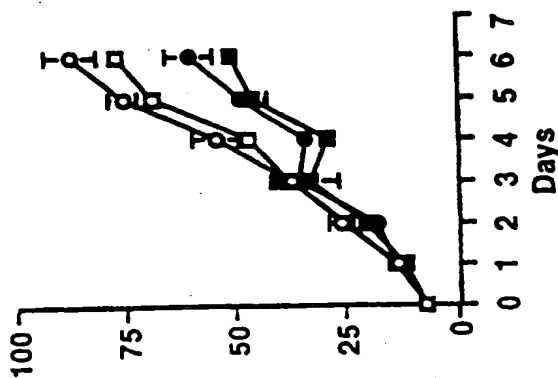


FIGURE 11B

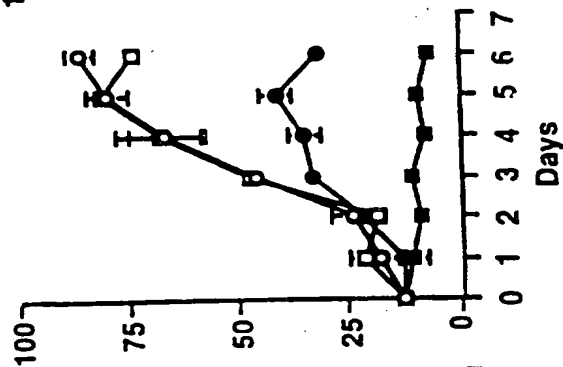
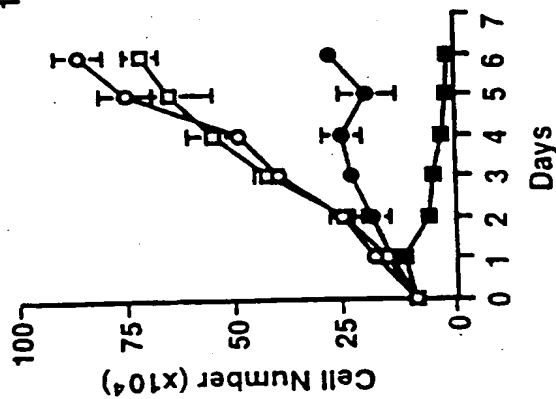


FIGURE 11A



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FIGURE 12B

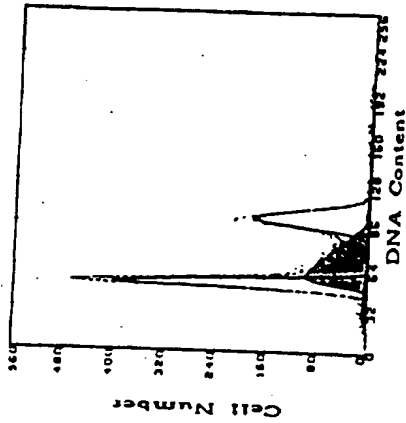


FIGURE 12D

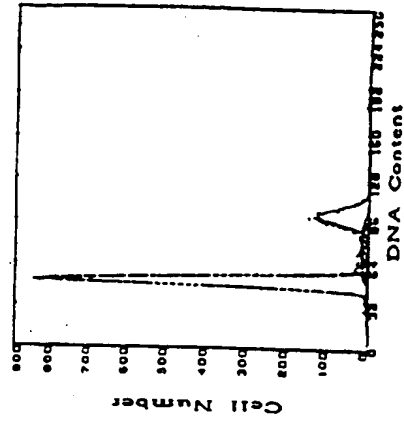


FIGURE 12A

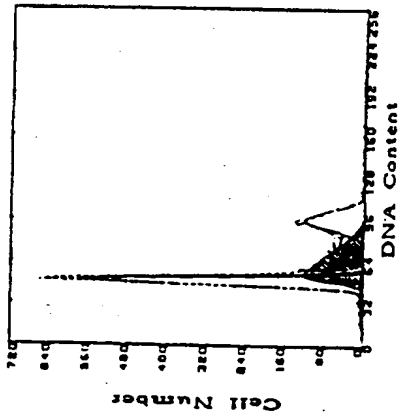
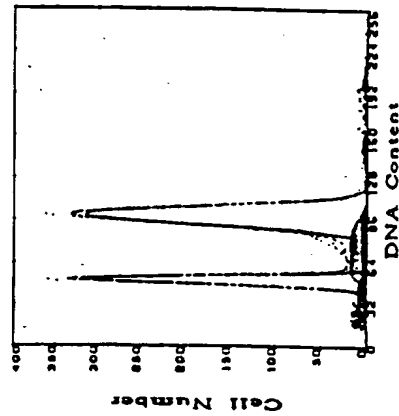


FIGURE 12C



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FIGURE 13A

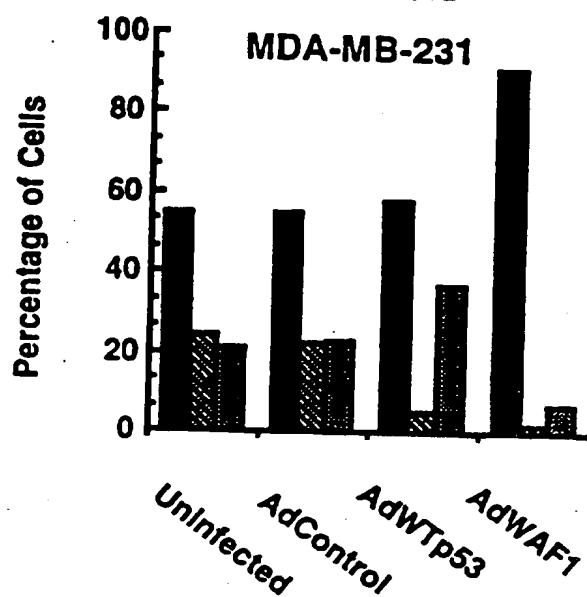


FIGURE 13B

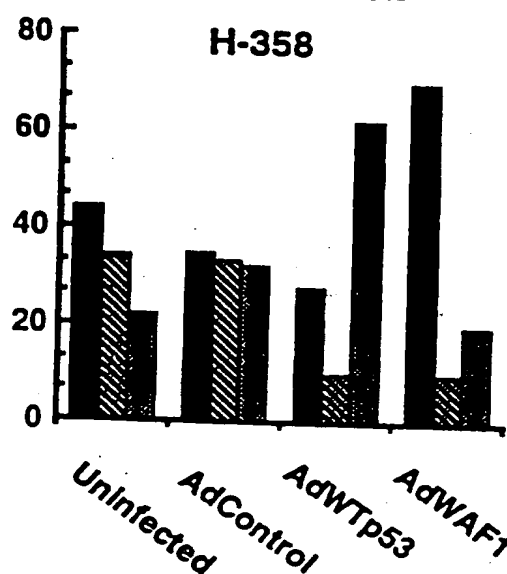


FIGURE 13C

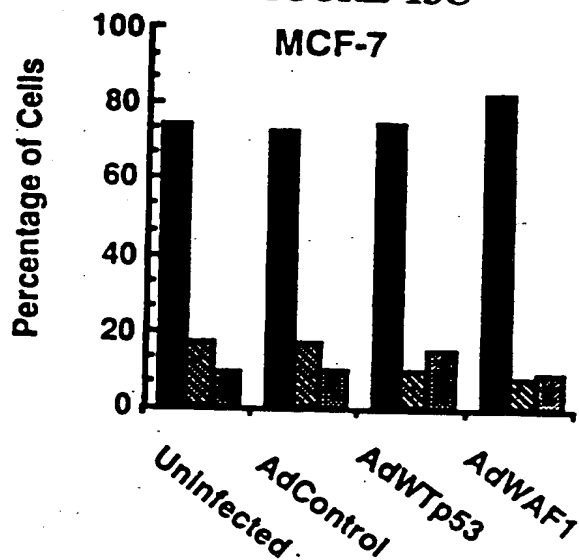
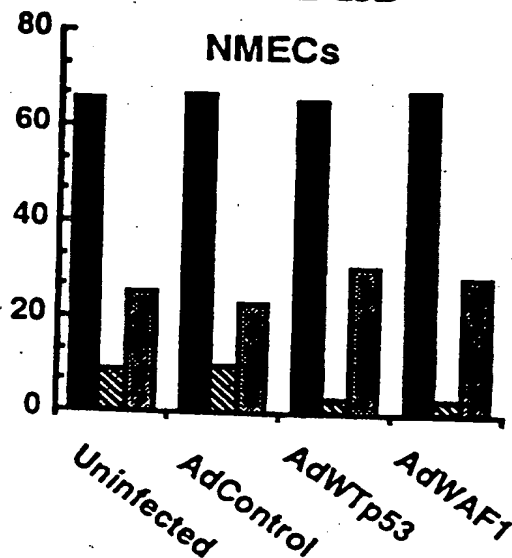


FIGURE 13D



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FIGURE 14A

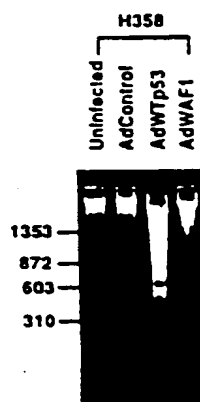


FIGURE 14B



FIGURE 14C

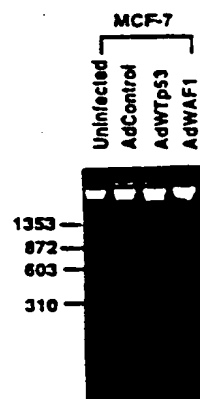


FIGURE 14D

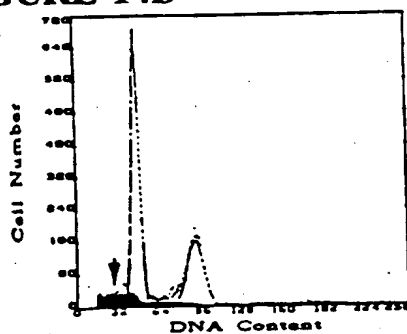
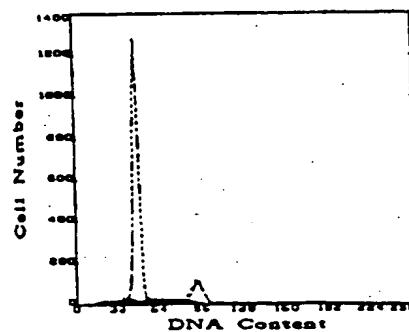
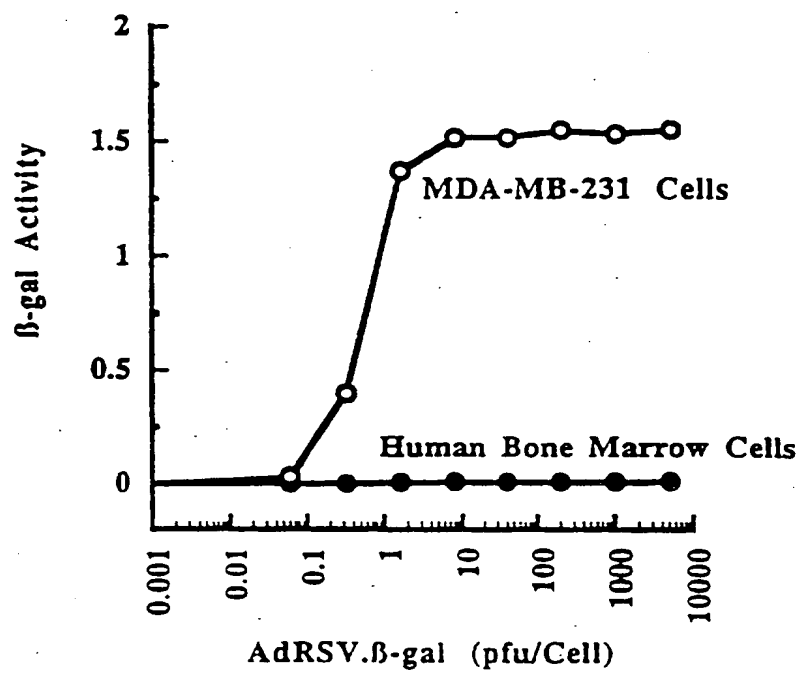


FIGURE 14E



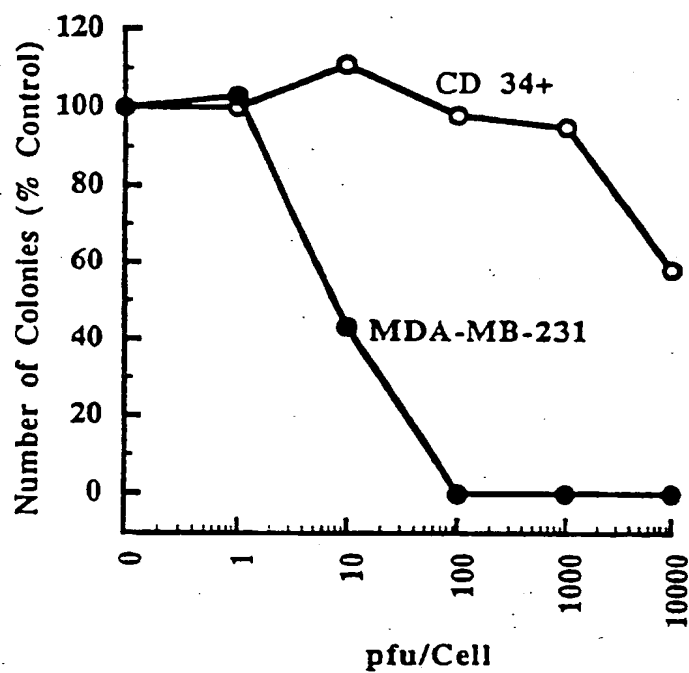
15 / 4 1

FIGURE 15



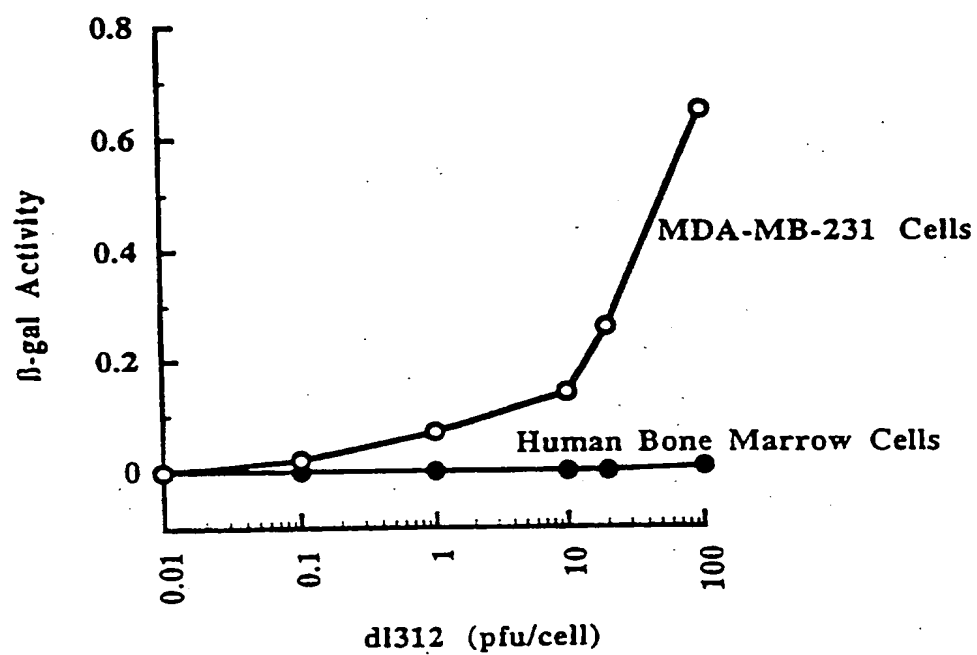
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FIGURE 16



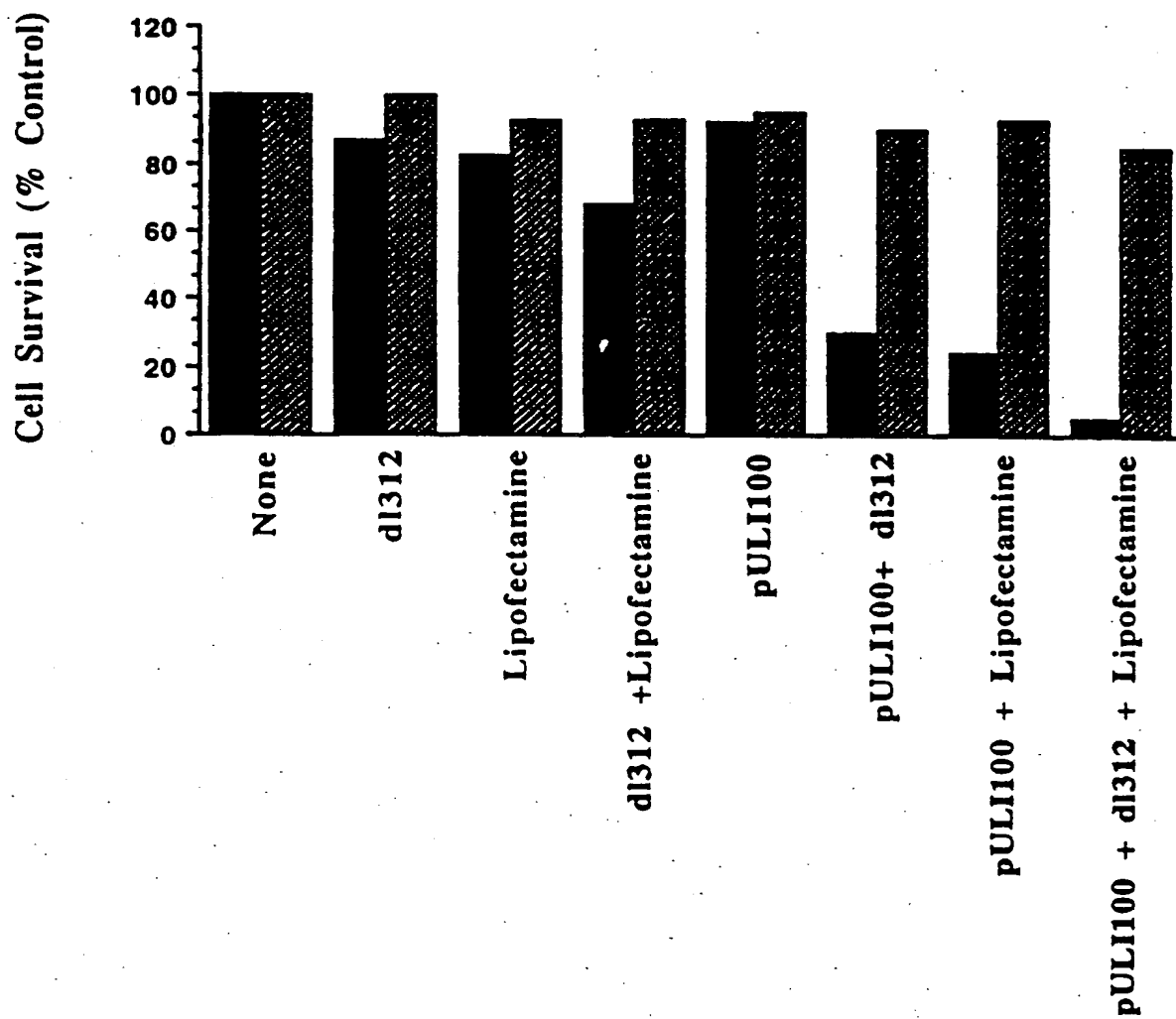
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FIGURE 17



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FIGURE 18



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Figure 19B

AdWTP53

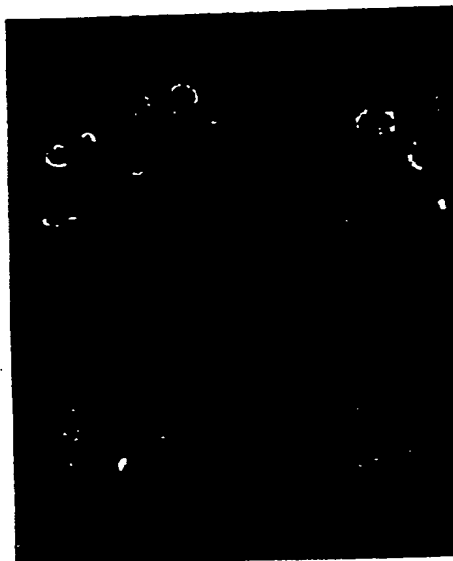
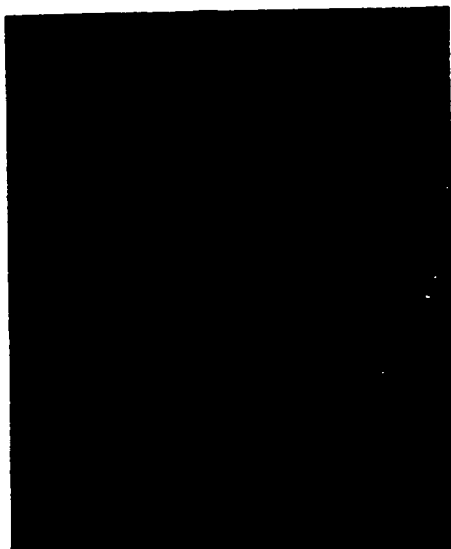


Figure 19A

AdControl



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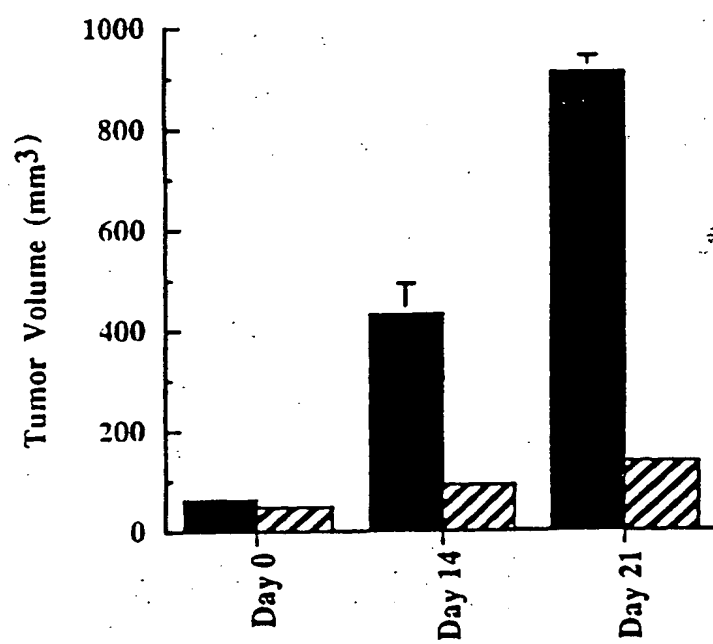
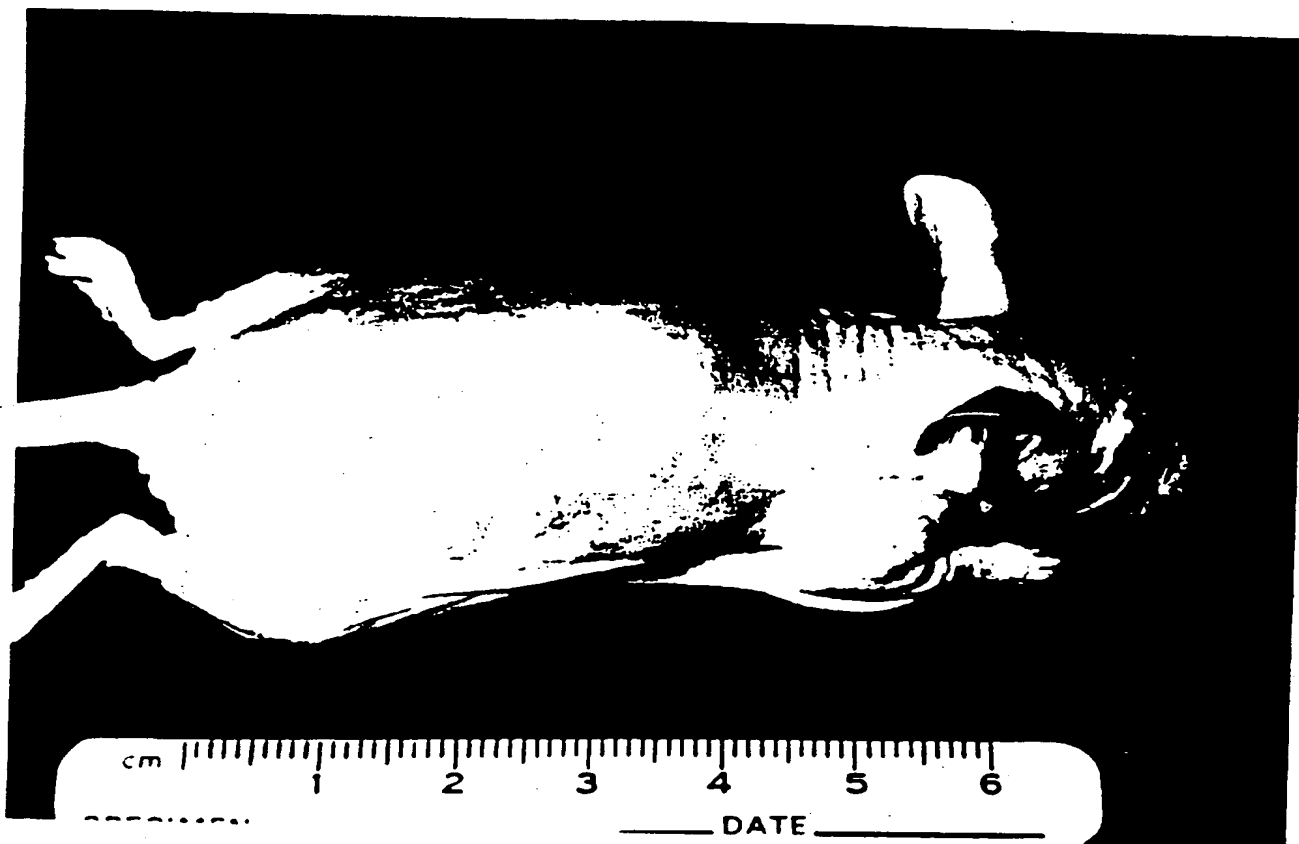


Figure 20

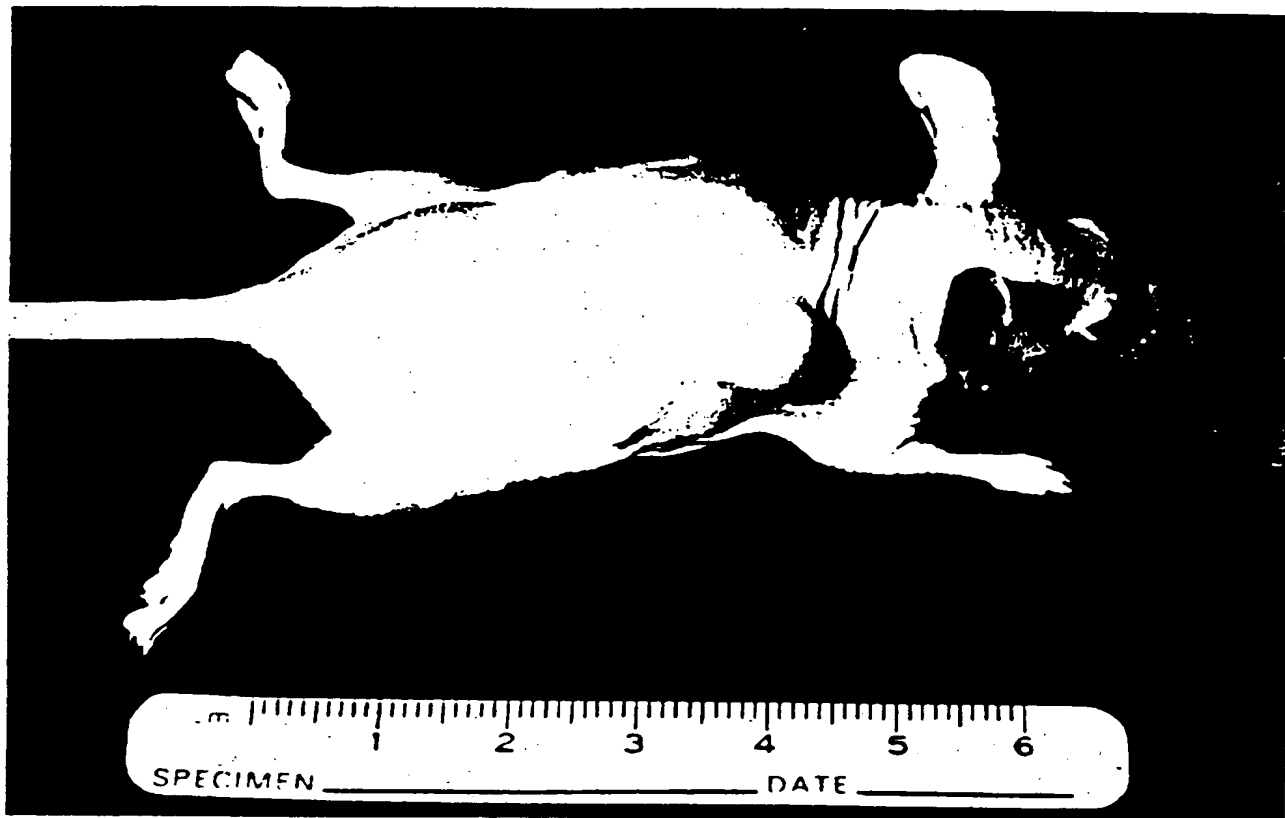
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Figure 21A



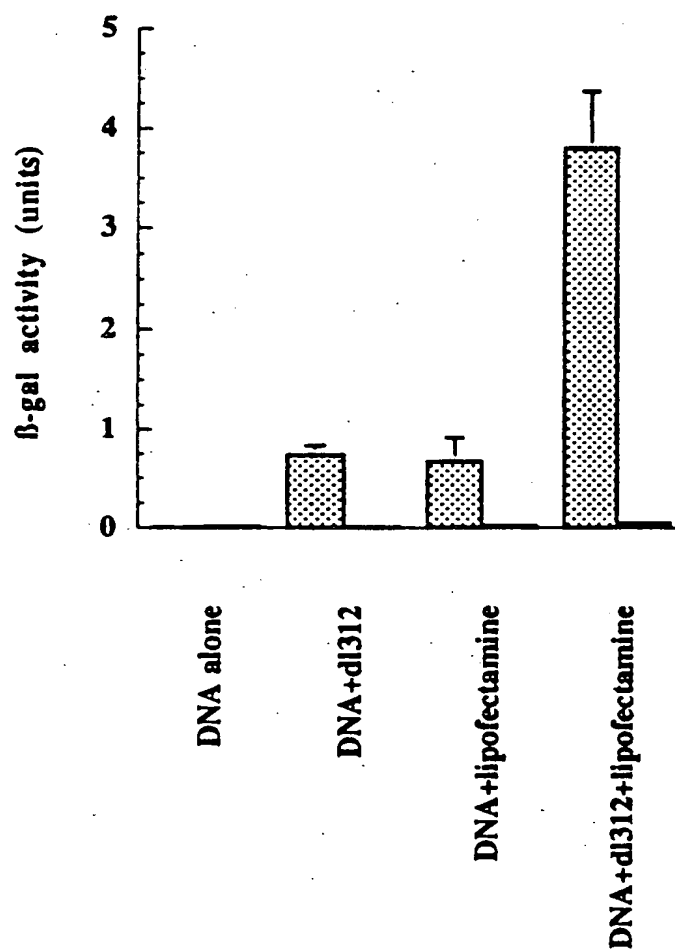
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Figure 21B

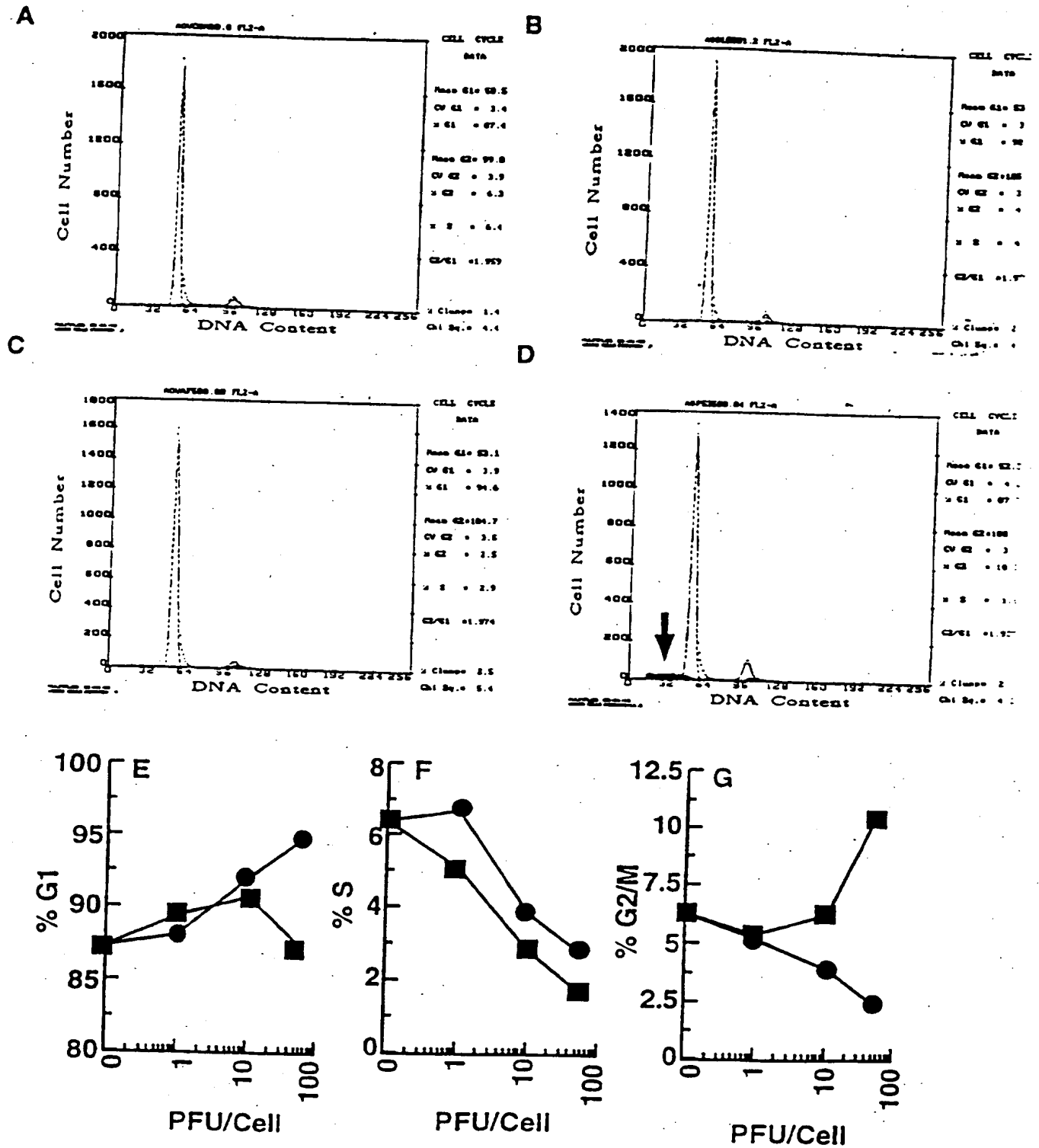


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Figure 22



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Figur 23A-23G

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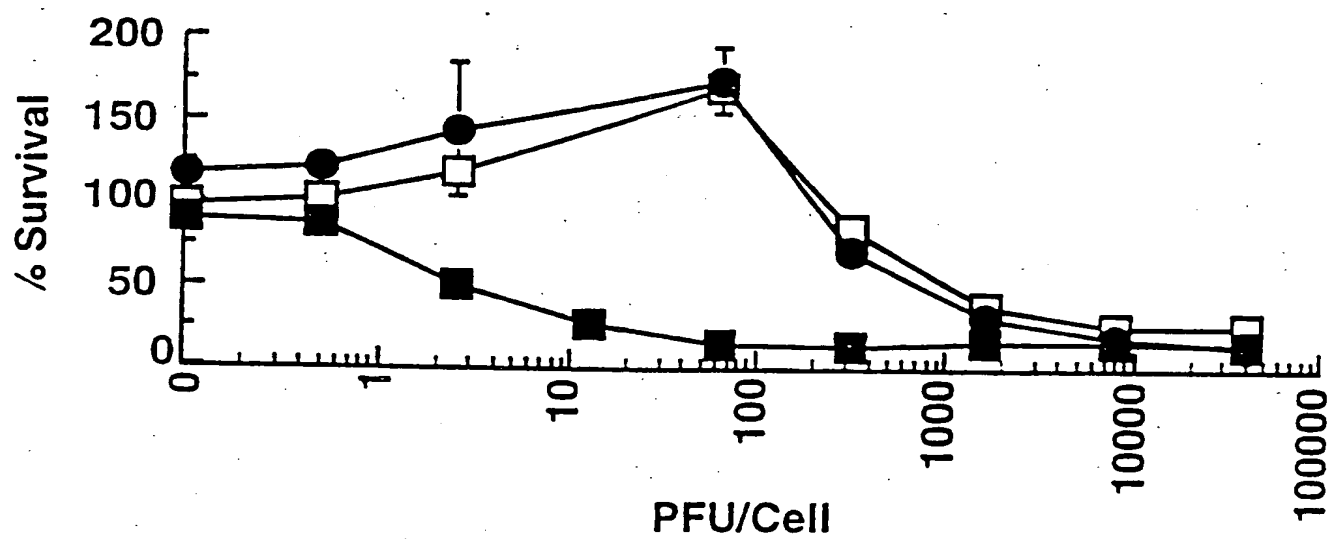


Figure 24

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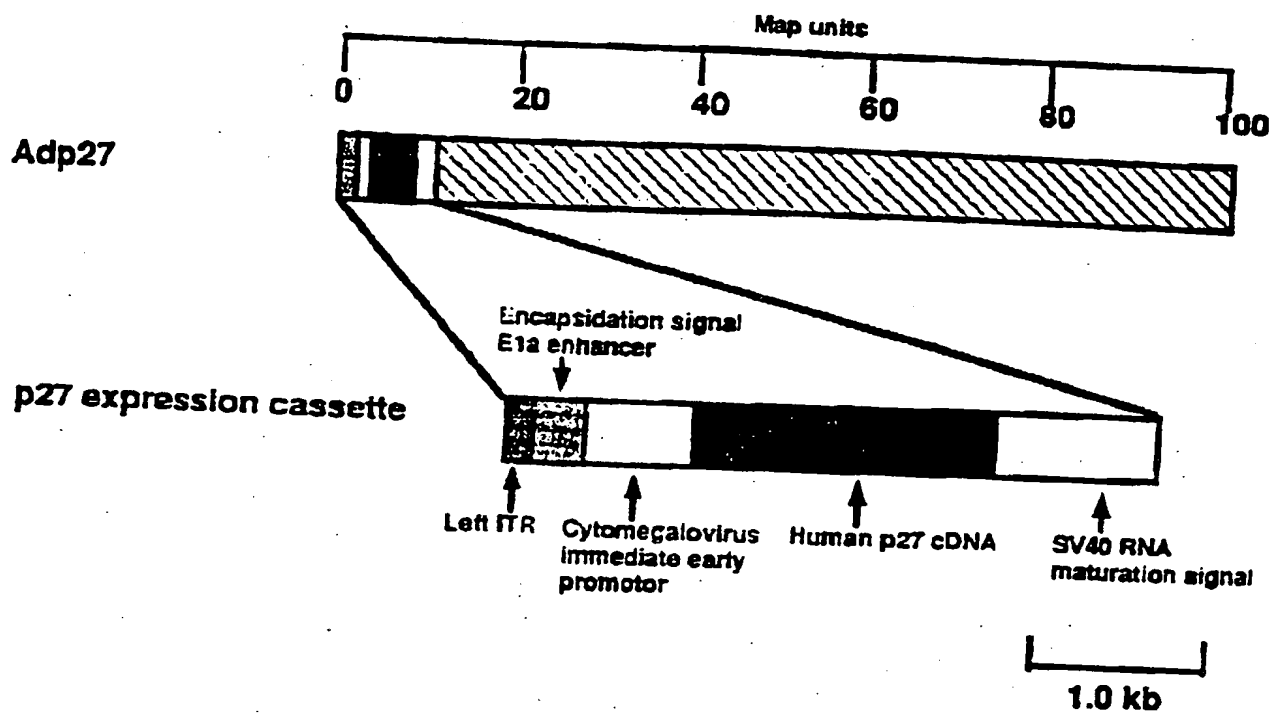


Figure 25

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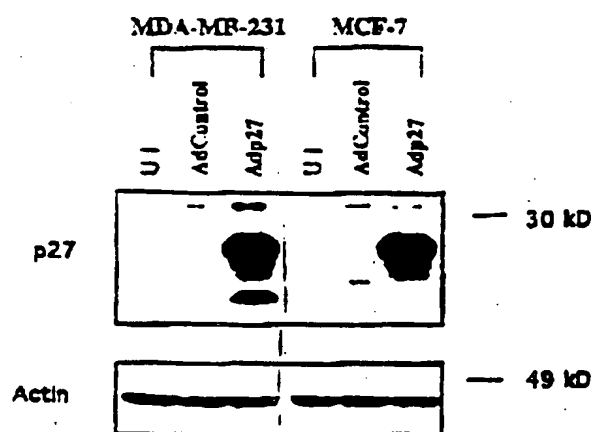


Figure 26

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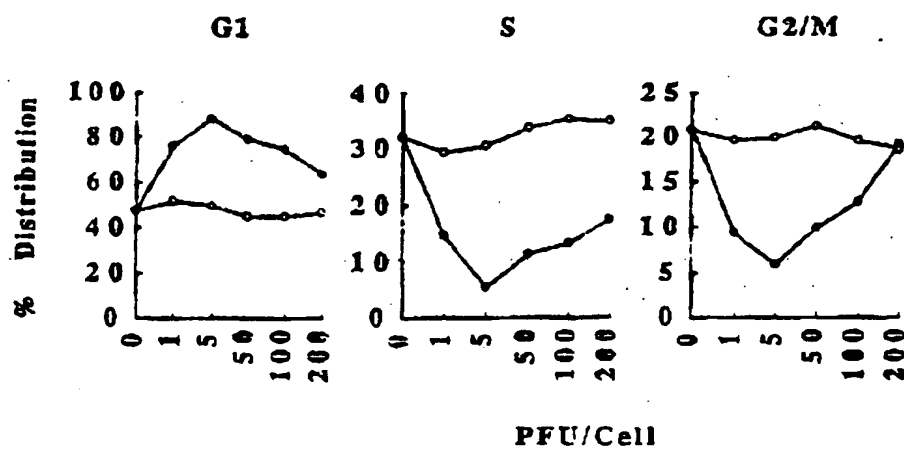


Figure 27A

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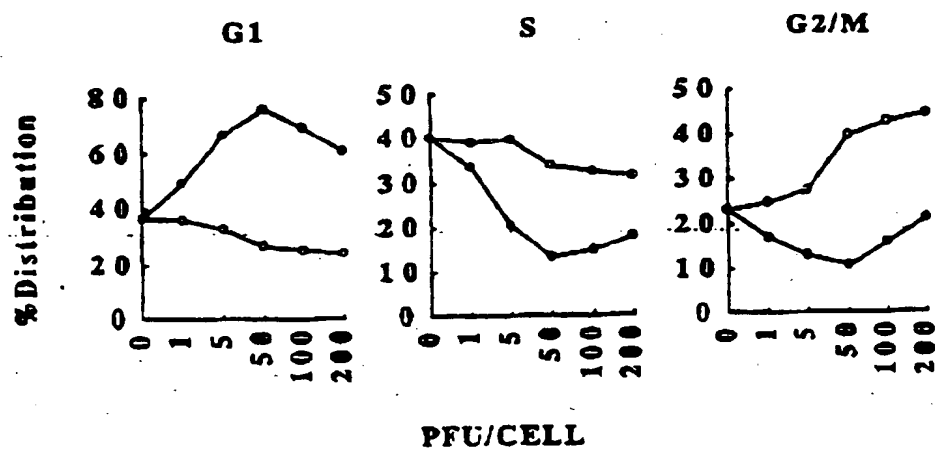


Figure 27B

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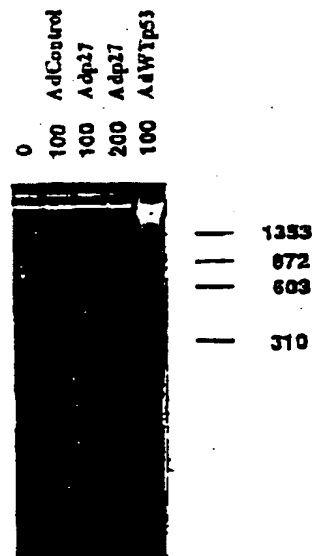


Figure 28

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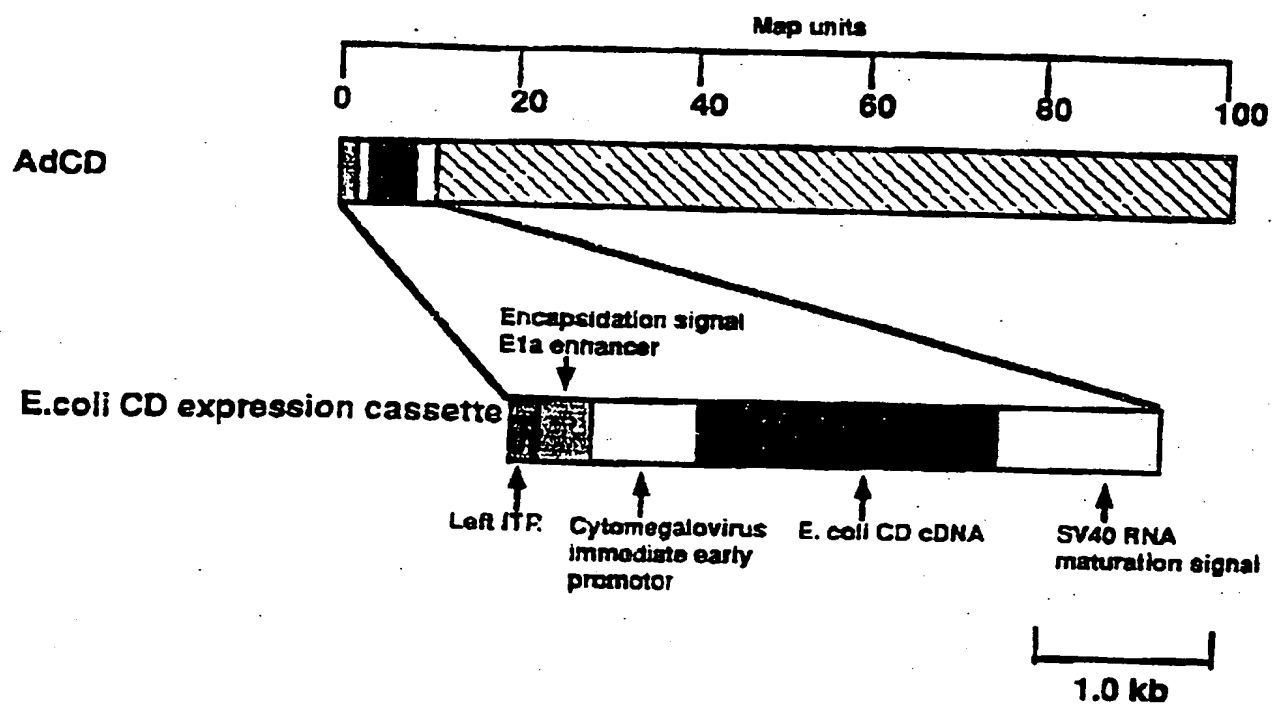


Figure 29

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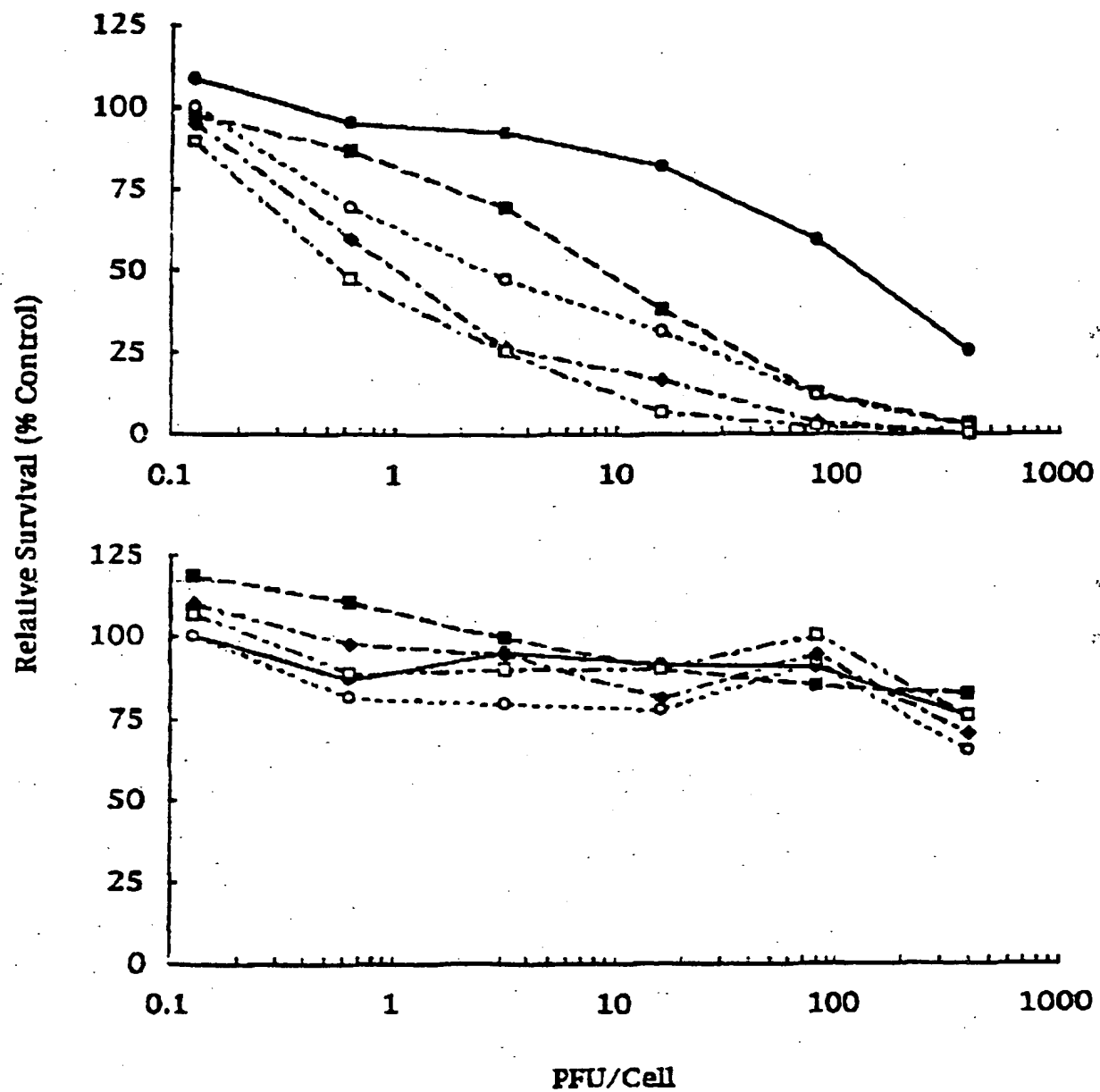
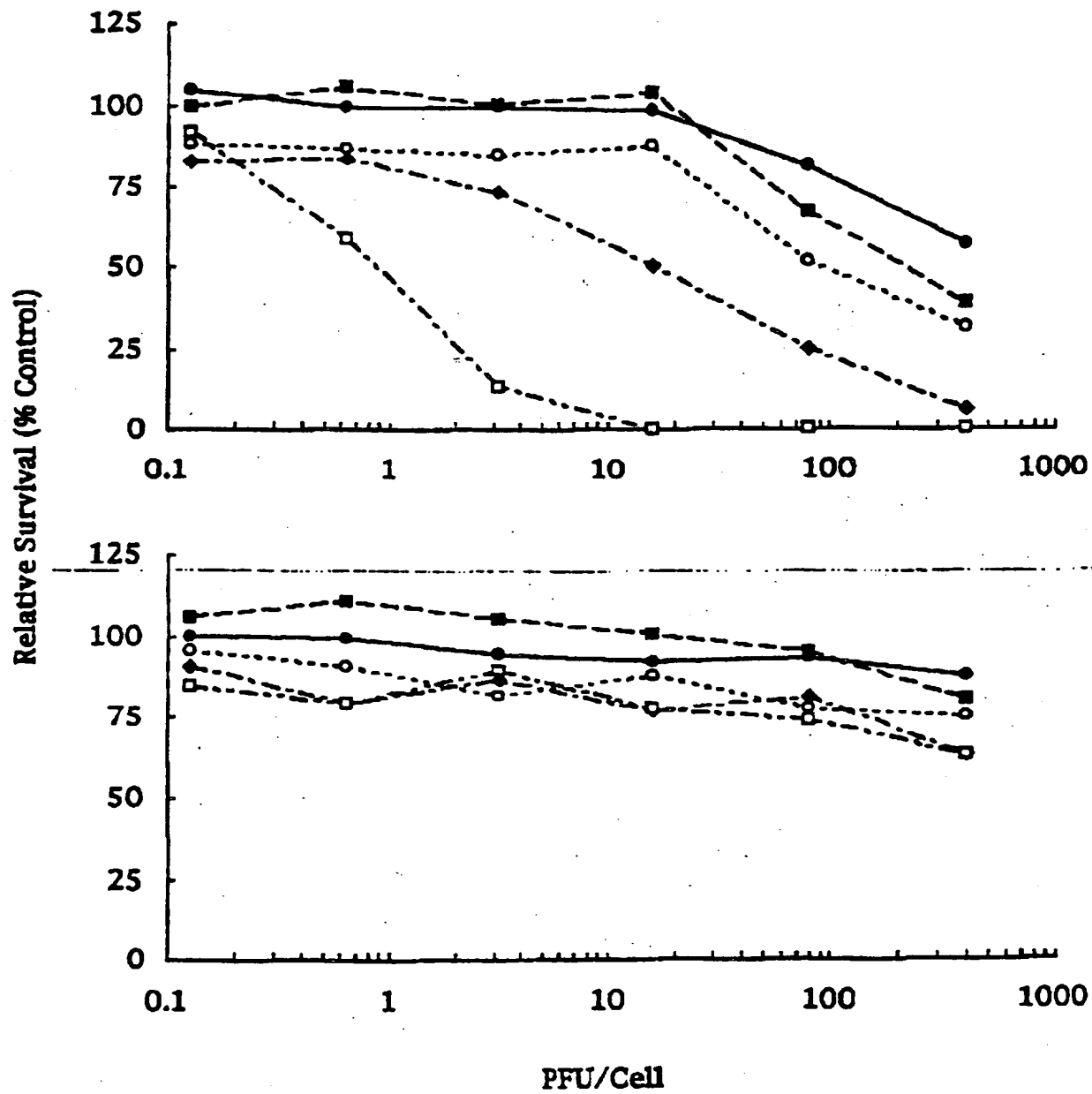


Figure 30A

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Figur 30B

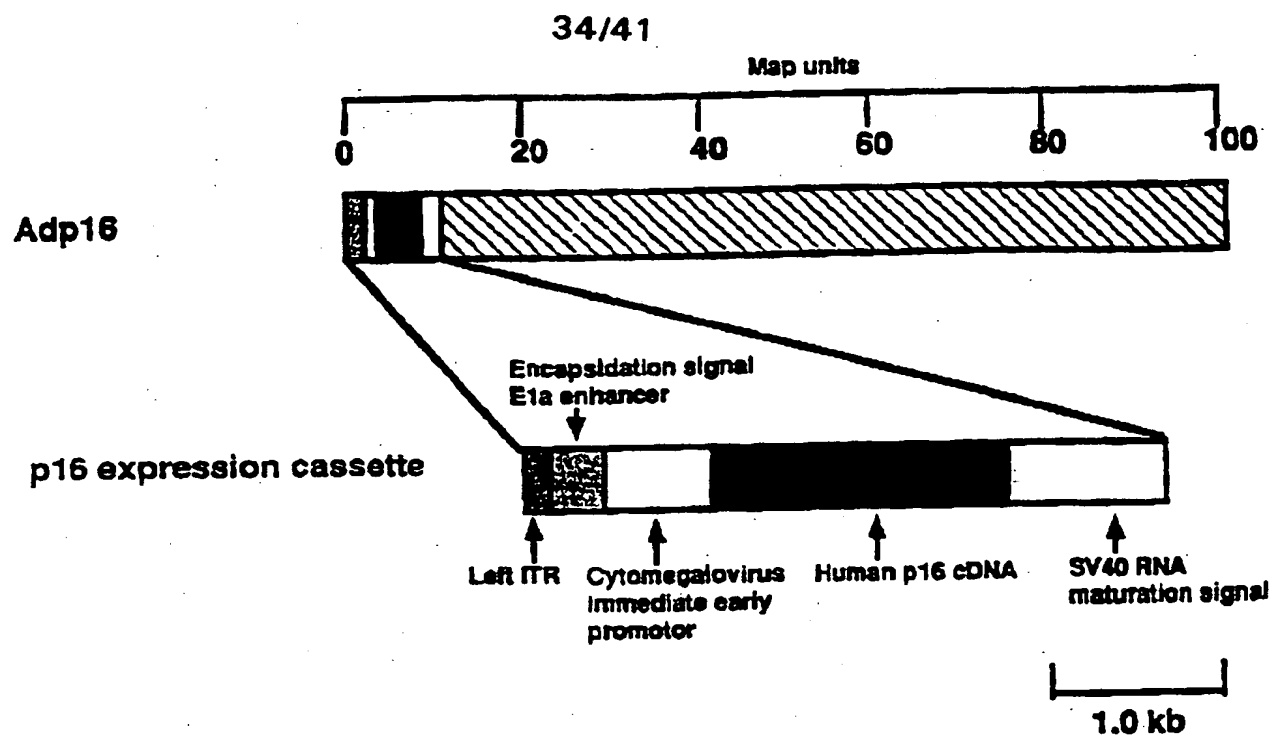


Figure 31

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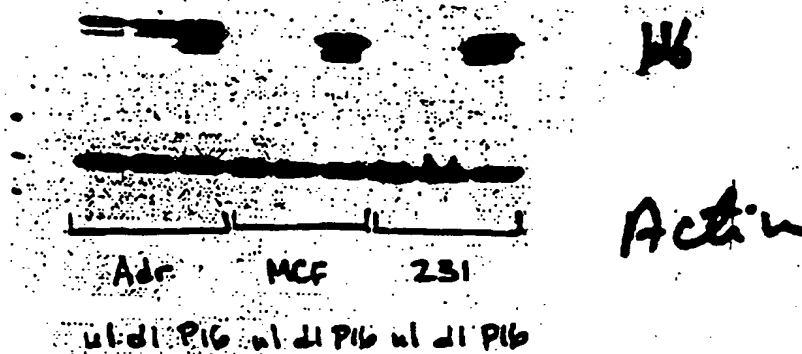


Figure 32

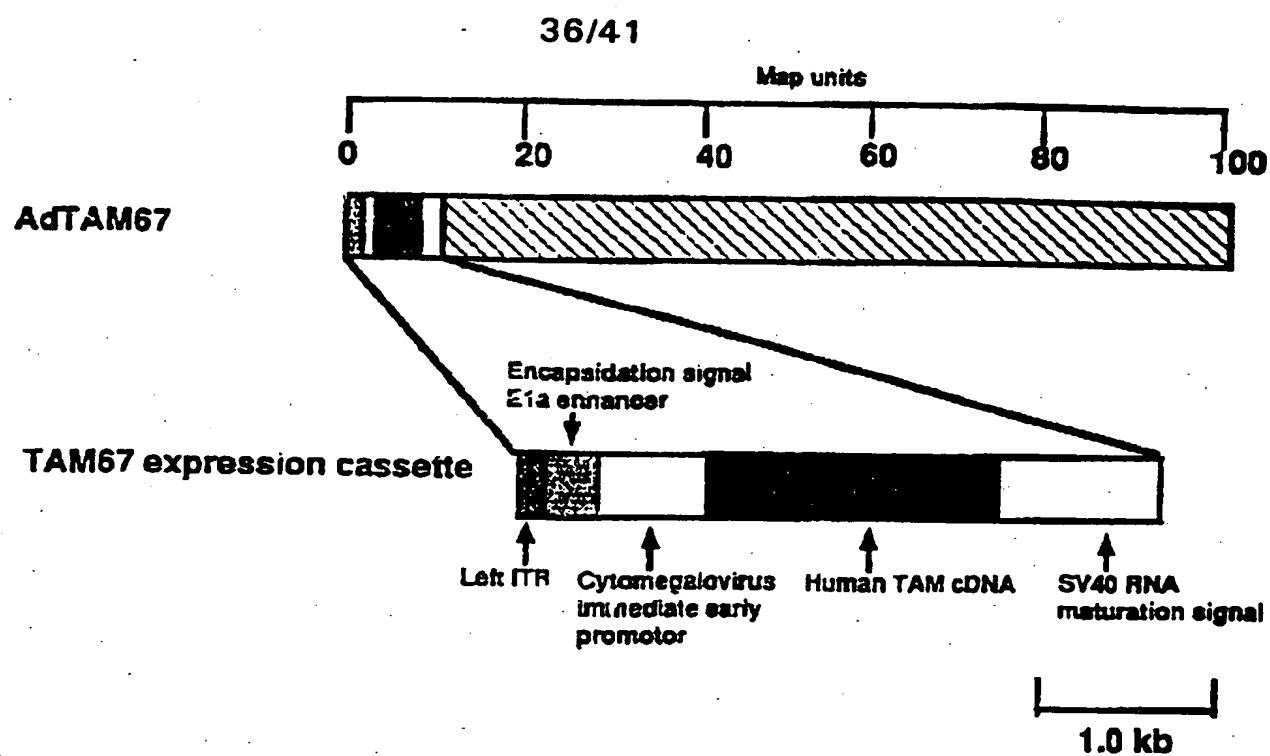


Figure 33

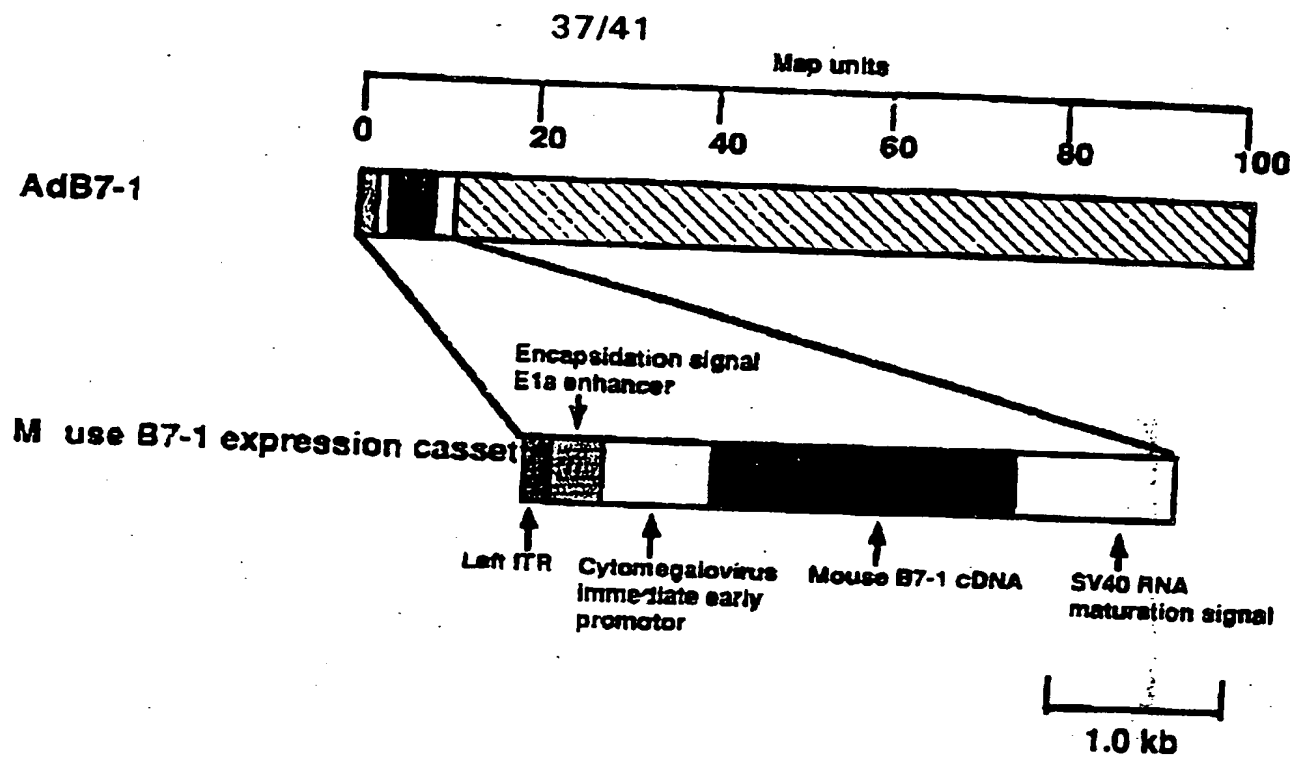


Figure 34

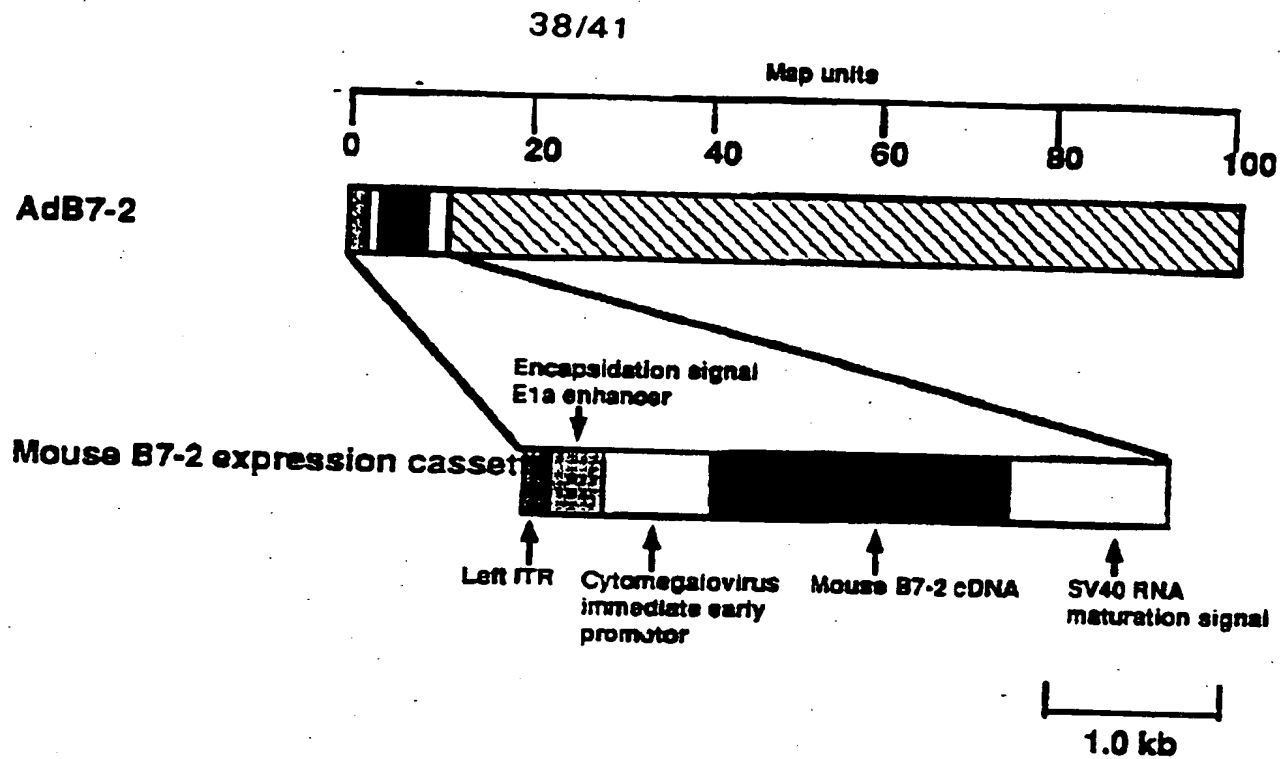


Figure 35

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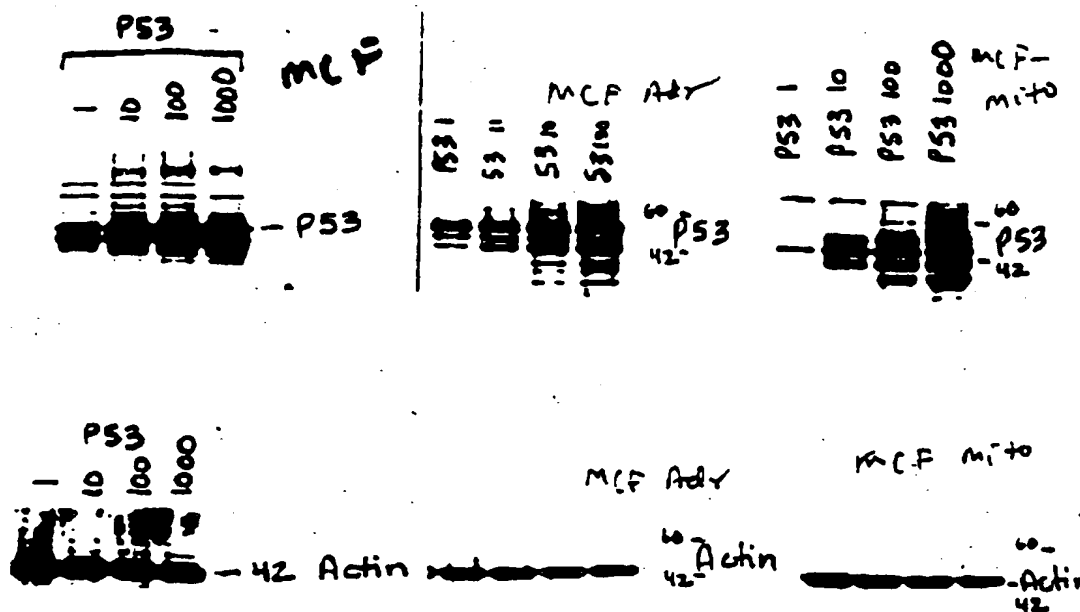
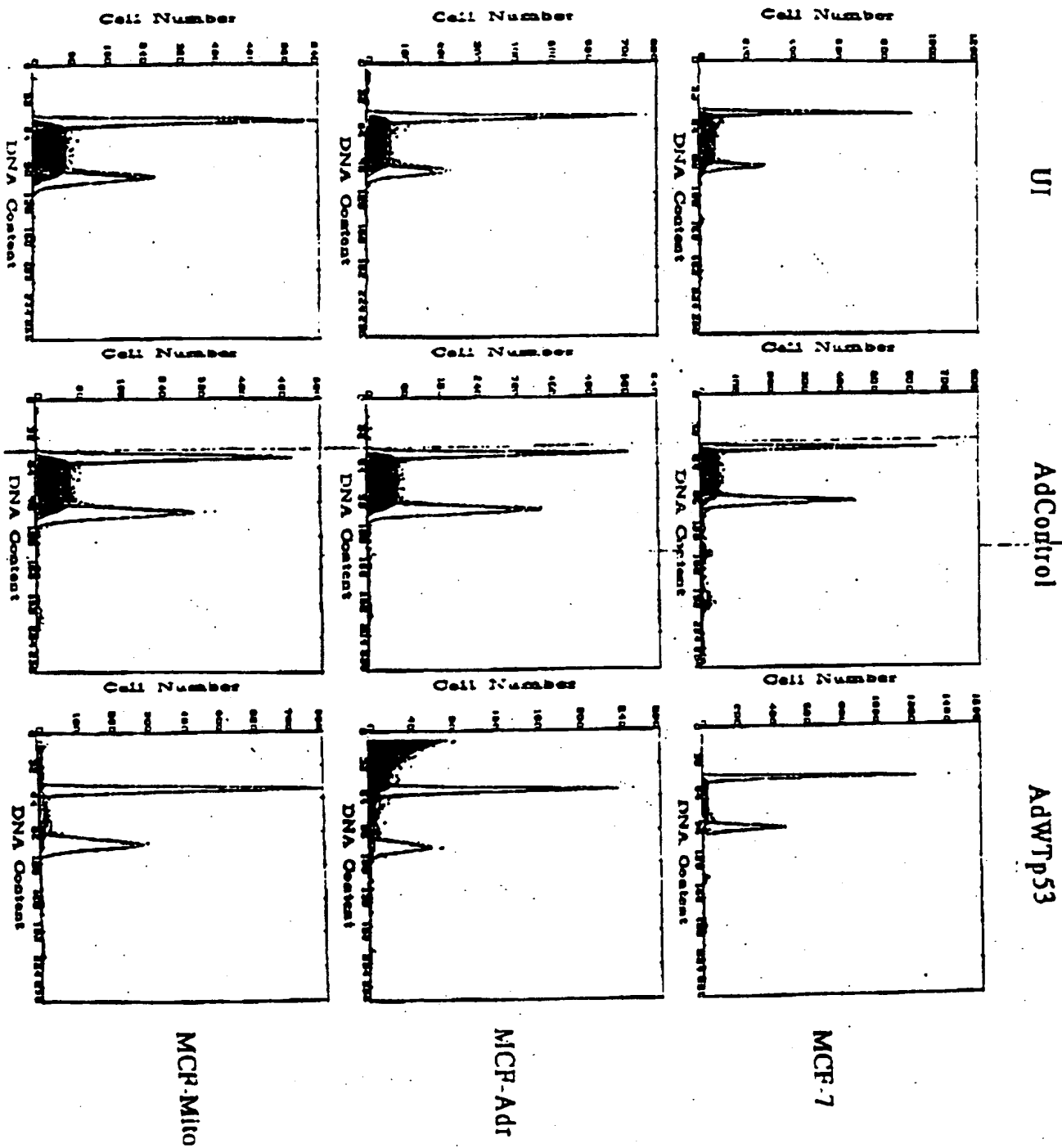


Figure 36

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Figure 37



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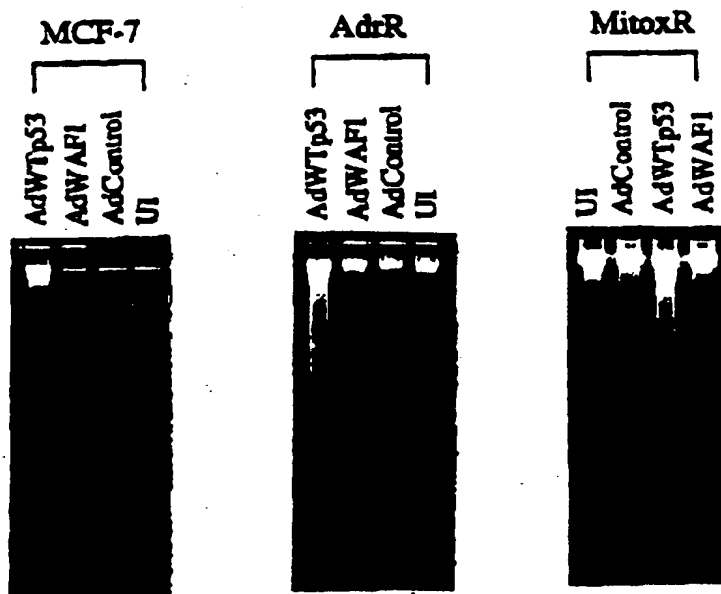


Figure 38



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, A61K 48/00, C07K 14/47, 14/82, C12N 15/12, 9/78, 15/55		A3	(11) International Publication Number: WO 96/25507
			(43) International Publication Date: 22 August 1996 (22.08.96)
(21) International Application Number: PCT/US96/02336 (22) International Filing Date: 16 February 1996 (16.02.96) (30) Priority Data: 08/390,604 17 February 1995 (17.02.95) US (60) Parent Application or Grant (63) Related by Continuation US 08/390,604 (CIP) Filed on 17 February 1995 (17.02.95) (71) Applicant (for all designated States except US): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SETH, Prem, K. [IN/US]; 11705 Cherry Grove Drive, North Potomac, MD 20878 (US). COWAN, Kenneth [US/US]; 12005 Gregersaroft Road, Potomac, MD 20854 (US).		(74) Agent: FEILER, William, S.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. (88) Date of publication of the international search report: 7 November 1996 (07.11.96)	
(54) Title: METHODS OF PREPARATION AND USE OF RECOMBINANT ADENOVIRAL VECTORS			
(57) Abstract			
<p>The present invention describes novel methods of constructing recombinant adenoviral vectors capable of expressing human cDNAs, such as wild-type p53, WAF1/Cip1/p21, p27/kip1, <i>E. coli</i> cytosine deaminase, wild-type p16, TAM 67 (a jun/fos dominant negative mutant) and B7-1 and B7-2. The invention further provides methods of inhibiting the proliferation of cells, inhibiting the cell cycle of proliferating cells, and methods for the eradication of cells, especially cancer and diseased cells, by infecting the cells with a recombinant adenovirus vector capable of expressing human cDNAs. Compositions and methods of the invention are suitable for treatment of a subject afflicted with a tumor wherein the cells of the tumor, for example, lack the wild-type p53 allele and/or possess a mutated p53 gene. The invention additionally provides a method for the use of adenoviral vectors in the treatment of cancer cells, such as lung cancer and breast cancer cells. The invention further provides methods for the use of adenoviral vectors in cancer gene therapy as a mechanism for purging bone marrow cells of contaminating tumor cells, for eradicating cancer cells, and for preventing development of cancer cells and tumors.</p>			

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INTERNATIONAL SEARCH REPORT

National Application No.

CT/US 96/02336

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 A61K48/00 C07K14/47 C07K14/82 C12N15/12
C12N9/78 C12N15/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRENDS IN BIOTECHNOLOGY, vol. 8, no. 4, 1990, pages 85-87, XP002012682 F. GRAHAM: "Adenoviruses as expression vectors and recombinant vaccines" see figure 2 ---	8-15
X	IN VIVO, vol. 8, 1994, pages 755-770, XP002012683 W. ZHANG ET AL.: "Anti-oncogene and tumour suppressor gene therapy - examples from a lung cancer model" see page 762 - page 765 --- -/--	8,10-15, 23-27, 29-46

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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12 September 1996

Date of mailing of the international search report

20.09.96

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NL - 2280 HV Rijswijk
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Authorized officer

Skelly, J

INTERNATIONAL SEARCH REPORT

International Application No

US 96/02336

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 24297 (PERRICAUDET) 27 October 1994 see the whole document ---	8,10-15, 23-27, 29-46
E	WO,A,96 15245 (ARCH DEVELOPMENT CORPORATION) 23 May 1996 see example 5 ---	8-15,23, 32
X,P	WO,A,95 13375 (THE JOHNS HOPKINS UNIVERSITY) 18 May 1995 see the whole document ---	8-13,15, 16,23-46
X,P	WO,A,95 10623 (GOVERNMENT OF THE UNITED STATES OF AMERICA) 20 April 1995 see page 9, line 6-13 ---	8,10-15, 23-27, 29-46
X,P	WO,A,95 18824 (SLOAN KETTERING INSTITUTE FOR CANCER RESEARCH) 13 July 1995 see page 34 - page 35 ---	9,10,15, 17,23-46
X,P	CANCER RESEARCH, vol. 55, 1995, pages 3250-3253, XP002012684 X. JIN ET AL: "Cell cycle arrest and inhibition of tumour cell proliferation by the p16ink4 gene mediated by an adenovirus vector" see the whole document ---	1-15,19, 23-46
X,P	BIOCHEM. BIOPHYS. RES. COMMUN., vol. 215, no. 2, 1995, pages 446-451, XP002012685 D. KAYATOSE ET AL.: "Consequences of p53 gene expression by adenovirus vector on cell cycle arrest and apoptosis in human aortic vascular smooth muscle cells" see the whole document ---	1-16, 23-46
X,P	CLINICAL CANCER RESEARCH, vol. 1, 1995, pages 889-897, XP002012686 D. KAYATOSE ET AL.: "Cytotoxic effects of adenovirus-mediated wild-type p53 protein expression in normal and tumor mammary epithelial cells" see the whole document ---	1-15, 24-28
X,P	WO,A,95 12660 (UNIVERSITY OF TEXAS) 11 May 1995 see the whole document -----	8,10-15, 23-27, 29-46

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 02336

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 23-30 (partially as far as they concern an in vivo method) 32-42, are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

P/US 96/02336

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9424297	27-10-94	FR-A- 2704234	28-10-94
		AU-A- 6572194	08-11-94
		CA-A- 2158869	27-10-94
		EP-A- 0695360	07-02-96
		FI-A- 954966	18-10-95
		HU-A- 73464	28-08-96
		NO-A- 954132	17-10-95
		ZA-A- 9402778	09-01-95
WO-A-9615245	23-05-96	AU-A- 4502096	06-06-96
WO-A-9513375	18-05-95	NONE	
WO-A-9510623	20-04-95	AU-A- 8017394	04-05-95
WO-A-9518824	13-07-95	AU-A- 1525195	01-08-95
		AU-A- 2770695	16-02-96
		WO-A- 9602140	01-02-96
WO-A-9512660	11-05-95	AU-A- 8094994	23-05-95
		CA-A- 2174556	11-05-95
		EP-A- 0725791	14-08-96
		NO-A- 961696	26-06-96



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, A61K 48/00, C07K 14/47, 14/82, C12N 15/12, 9/78, 15/55		A3	(11) International Publication Number: WO 96/25507
			(43) International Publication Date: 22 August 1996 (22.08.96)
(21) International Application Number: PCT/US96/02336		(74) Agent: FEILER, William, S.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).	
(22) International Filing Date: 16 February 1996 (16.02.96)			
(30) Priority Data: 08/390,604 17 February 1995 (17.02.95) US		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Application or Grant (63) Related by Continuation US 08/390,604 (CIP) Filed on 17 February 1995 (17.02.95)		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for all designated States except US): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).		(88) Date of publication of the international search report: 7 November 1996 (07.11.96)	
(72) Inventors; and (75) Inventors/Applicants (for US only): SETH, Prem, K. [IN/US]; 11705 Cherry Grove Drive, North Potomac, MD 20878 (US). COWAN, Kenneth [US/US]; 12005 Gregersaroff Road, Potomac, MD 20854 (US).			
(54) Title: METHODS OF PREPARATION AND USE OF RECOMBINANT ADENOVIRAL VECTORS			
(57) Abstract			
<p>The present invention describes novel methods of constructing recombinant adenoviral vectors capable of expressing human cDNAs, such as wild-type p53, WAF1/Cip1/p21, p27/kip1, <i>E. coli</i> cytosine deaminase, wild-type p16, TAM 67 (a jun/fos dominant negative mutant) and B7-1 and B7-2. The invention further provides methods of inhibiting the proliferation of cells, inhibiting the cell cycle of proliferating cells, and methods for the eradication of cells, especially cancer and diseased cells, by infecting the cells with a recombinant adenovirus vector capable of expressing human cDNAs. Compositions and methods of the invention are suitable for treatment of a subject afflicted with a tumor wherein the cells of the tumor, for example, lack the wild-type p53 allele and/or possess a mutated p53 gene. The invention additionally provides a method for the use of adenoviral vectors in the treatment of cancer cells, such as lung cancer and breast cancer cells. The invention further provides methods for the use of adenoviral vectors in cancer gene therapy as a mechanism for purging bone marrow cells of contaminating tumor cells, for eradicating cancer cells, and for preventing development of cancer cells and tumors.</p>			

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ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

**METHODS OF PREPARATION AND USE OF
RECOMBINANT ADENOVIRAL VECTORS**

FIELD OF THE INVENTION:

5 The invention describes novel methods of constructing
recombinant adenoviral vectors capable of expressing human
cDNAs, and methods of use for inhibiting proliferation of
cells and methods of eradicating cells. Compositions and
methods of the invention are suitable for treatment of a
10 subject afflicted with a tumor and are also useful in cancer
gene therapy as a mechanism for purging bone marrow cells of
contaminating tumor cells, and preventing the development of
cancer cells and tumors.

BACKGROUND OF THE INVENTION:

15 In recent years, recombinant adenoviruses have become
a popular tool for the study of both adenoviral biology and
in vitro and in vivo gene transfer. For gene therapy
purposes, adenoviral vectors have been rendered replication-
deficient by replacing the replication regulating Ela
20 nucleotide sequence located at the 5' end of the adenovirus
genome with foreign gene expression cassettes.

To replace the portion of the Ela nucleotide sequence
in the adenoviral genome with the foreign gene expression
cassette, one must modify the adenoviral genome so that
25 adenovirus virions are not produced. These modifications
are known in the art, and have been achieved through the
application of two methods. One method utilizes the
presence of a unique ClaI restriction site to excise 900
base pairs of the 5' end of the adenovirus genome (2.6 map
30 units of the adenovirus genome). The remaining nucleotide
sequence of the adenovirus genome, devoid of its' 5' end,
does not produce virions as it has lost two essential
elements critical for the replication of adenovirus genome:
(1) the left inverted terminal repeat; and (2) half of the
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Ela sequences. Perricaudet and their colleagues pioneered this single ClaI digestion technique (mainly using genomic DNA of d1327 mutant of Ad5) and succeeded in constructing adenoviral vectors. (See Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M. and Briand, P. J., J. Clin. Invest. 90:626-630 (1992)).

However, the single ClaI digestion technique poses several problems. Initially, there are difficulties in the isolation of recombinant adenovirus due to the high background of parental genome (d1327). When d1327 DNA is cut with ClaI restriction enzyme, uncut DNA still remains. Because the difference between the genomic DNA sizes of the desired (32 kb) and undesired (33 kb) DNA is only about 1 kb, it is virtually impossible to separate the two DNA fragments derived from the partial digestion of the adenovirus genome. To circumvent this problem, one must screen a large number of possible recombinant adenoviruses to rescue a single recombinant adenovirus.

Another approach for modification of the adenovirus genome is by the replacement of DNA sequences in the adenoviral genome by another DNA fragment, resulting in a adenoviral genomic sequence large enough to exceed the packaging limit of the adenovirus virions. This problem has been addressed through a method which replaces the 2.2 kb fragment of pFG140 (a circular DNA derived from d1309 genome with 4.4-kb DNA fragment) containing an ampicillin resistance gene and a bacterial origin of replication (See Graham, F. L. and Prevec, L. (1991) Manipulation of adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene transfer and expression protocols, Humana Press, Clifton, New Jersey)). The resulting plasmid, designated pJM17, may then be propagated as a plasmid. pJM17 can be rescued as infectious virions when the foreign 4.4 kb fragment of pJM17 is replaced by homologous recombination with another DNA fragment small enough for the resulting genome to package the adenovirus virion.

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Although the pJM17 system solves the background problem present during the screening of recombinant adenoviruses, the difficulty in the quality control of the plasmids and the recombinant vectors still remains. This may be due to the large size and structure of pJM17. pJM17 is a very large circular DNA molecule (about 40 kb) and has a tendency to undergo undefined rearrangements. After such homologous recombination, specific DNA sequences are occasionally kicked out from the recombinant constructs in vivo, thus rendering the expression of the DNA inserts impossible.

The present invention provides a novel method for the construction of adenoviral vectors. The invention provides a technique of constructing an adenoviral vector whereby an additional ClaI restriction site is introduced upstream of the original ClaI site. The introduction of the second ClaI restriction site greatly reduces the chances of obtaining undigested DNA genome. Because only one of the two ClaI sites must be cut to prevent the production of the non-recombinant background infectious virions, the addition of a second ClaI site greatly increases the chances of generating fully cut DNA and thus reducing the parental genome background. Moreover, because the adenoviral vectors are made using viral genomic DNA as the starting point, there is no need to utilize a plasmid based vector.

Adenoviral vectors are generally the preferred vector for the expression of DNA fragments. Although plasmids and retroviruses have been used to express DNA fragments, the efficiency of transfection is generally low. (See Chen, et al., Science, 250:1576-1579, (1990); Shaw, et al., Proc. Natl. Acad. Sci. USA, 89:4495-4499, (1992); and Casey, et al., Oncogene, 6:1807-1811, (1991)). Adenoviral vectors are the preferred vector because they possess certain characteristics which allow for a high efficiency of transfection. Adenovirus based vectors are capable of a high efficiency of transfections because 1) they can grow to high titers; 2) they are internalized into cells with an

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efficient receptor-mediated endocytosis; 3) are replication incompetent; and 4) they express a transgene to high levels in epithelial cells. (Ginsberg, H. S., *Virology*, eds. Dulbecco, R. and Ginsberg, H. S., (Lippincott, Co., Philadelphia) pp 147-160 (1988); Graham, et al., In *Gene transfer and expression protocols*, (Murray, E. J. ed.) pp. 109-128, Humana Press, Clifton, New Jersey (1991); Seth, et al., *Virus attachment and entry into cells*, eds. Colwell, R. L., and Lonberg-Holm, K. (American Society for Microbiology, Washington, D.C.) pp 191-195, (1986); Seth, et al., *J. Virol.* 68:933-940 (1994); Rosenfeld, et al., *Hum. Gene Ther.* 5:331-342 (1994)).

Methods of constructing adenoviral vectors capable of expressing of cDNA fragments, such as wild-type p53, WAF1/Cip1/p21, wild-type p16, p27/kip1, and *E. coli* cytosine deaminase are provided by the present invention. The utilization of adenoviral expression vectors capable of producing high levels of proteins in cells allows for the study of the roles of these proteins in the control and regulation of cell growth in both normal and malignant cells. Furthermore, this strategy has implications in gene therapy for cancers.

It is commonly known that the protein encoded by the wild-type p53 gene affects cell proliferation by recognizing DNA damage to a cell, resulting in either a delay in progress through the cell cycle to allow for the repair processes of the cell to proceed, or by the initiation of programmed cell death, and/or the induction of apoptosis. See Kuerbitz, et al., *Proc. Natl. Acad. Sci. USA*, 89:7491-7495 (1992); Kastan, et al., *Cancer Res.*, 51:6304-6311, (1991); Lowe, et al., *Cell*, 74:957-968 (1993); and Levine, et al., *Br. J. Can.*, 69:409-416 (1994).

For those cells which express mutant p53, the effects of wild-type p53 are abrogated, resulting in abnormal cell growth and an increase the number of cells leading to cancer. Those cancers which have a high percentage of p53

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mutations and selection against the wild-type p53 protein are, among others, those arising in the colon, lung, bladder, prostate, breast, and skin. See Hollstein, et al., Science, 253:49-53, (1991); and Levine, et al., Nature, 253:453-456, (1991). Recently, there has been an increasing interest in elucidating the mechanisms by which p53 mediates its functions in normal cells, how various mutations in p53 are responsible for aberrant cell growth, and in the possibility of employing wild type p53 in gene therapy. See Nigro, et al., Nature (Lond.), 342:705-708 (1989); Chen, et al., Science, 250:1576-1579, (1990); and Gottesman, M.M., J. Natl. Cancer Inst., 86:1277-1285, (1994). It is therefore important to understand the biological consequences of overexpression of the wild-type p53 gene in both normal and tumor cells.

A number of approaches have been employed in the study of the effects of p53 expression in cells, including the exposure of cells to DNA-damaging agents such as ultraviolet radiation and chemicals that react with DNA, both of which have been shown to induce increased expression of cellular p53. See Clarke, et al., Nature, 362:849-852 (1993); Lowe, et al., Nature (Lond.), 362:847-849, (1993); and Dulic, et al., Cell, 76:1013-1023, (1994).

Genetic approaches have similarly been used to study the effects of p53 expression in cells. These approaches specifically include the introduction of temperature sensitive mutants of p53 or gene knock-out experiments to alter intracellular p53 expression and function. See Michalovitz, et al., Cell, 62:671-680, (1990); Chiou, et al., Mol. Cell. Biol., 14:2556-2563, (1994); and Donehower, et al., Nature 356:215-221, (1992).

It has been shown that p53 expression can transcriptionally activate several genes, including WAF1/Cip1/p21, which was also independently isolated as a negative growth regulatory gene. (Xlong, et al., Nature 366:701-704, (1993); El-Deiry, et al., Cell 75:817-825, (1993);

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Harper, et al., Cell 75:805-816, (1993)). Specifically, when DNA damage is induced, the intracellular levels of p53 rise and induce the expression of WAF1/Cip1. p21, the protein encoded by WAF1/Cip1, then binds to cyclin-dependent kinases and inhibits their activity. This event enables the cell cycle to be arrested before DNA synthesis, giving the cell the opportunity to repair the damaged DNA.

Thus, the utilization of an expression vector capable of producing high levels of WAF1/Cip1/p21 protein in cells would be useful in the determination of the exact relationship between p53 and WAF1-mediated cell growth regulation causing cell cycle arrest and/or apoptosis. Further, utilization of this vector would be useful in the determination of the effects of WAF1/Cip1/p21 gene expression in the absence of other p53-mediated signal transducing agents.

An additional area in which tools are needed for examination of the effects of proteins on cancer is in the area of vascular diseases. Vascular smooth muscle cells constitute the medial layer of arterial walls that maintains the normal tonus and resistance of vessels. The abnormality of vascular smooth muscle cells induces both functional and anatomical changes of the vessels (Ross, R. (1986) New Engl. J. Med. 314:488-500; Isoyama, S., et al., (1989) J. Clin. Invest. 84:288-294; and Ogata, M., et al. (1992) Am. J. Physiol. 262:H691-697), and these abnormalities could, in turn, give rise to a variety of serious stresses to the heart (Katayose, D., et al. (1993) Biochem. Biophys. Res. Commun. 191:587-594; Sandoval, J., et al., (1994) Circulation 89:1733-1744; and Tajima, M., et al., (1994) Cardiovasc. Res. 28:312-319). In anatomical changes of the vessel walls in vascular disease such as arteriosclerosis, pulmonary hypertension and vascular injury after angioplasty (Ross, R. (1986) New Engl. J. Med. 314:488-500; Botney, M.D., et al. (1994) Am. J. Pathol. 144:286-295; Katayose, D., et al., (1993) Am. J. Physiol. 264:L100-L106; and Speir,

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E., et al., (1994) Science 265:391-394), increased cellularity by vascular smooth muscle cell proliferation are observed. Given this knowledge, one of the possible strategies for controlling vascular diseases is to decrease the cellularity of vessel walls by letting vascular smooth muscle cells undergo cell cycle arrest and/or apoptosis.

Adenoviral vector constructs that would be useful in the understanding of the biochemical mechanisms underlying the cell cycle progression through various stages are those that express cyclin kinase inhibitors. A key contribution has been the cloning of several cyclin kinase inhibitors, such as p21/WAF1/Cip1, p27/kip1, and p16/INK4. Recent work has indicated that each one of these kinase inhibitors can potentially regulate one or more of the cyclin kinases leading to the dephosphorylation of Rb protein, which in turn can control the progression of the cell cycle into S phase. While other RB-like proteins such as p107 and p130 can also potentially control this signal transduction pathway leading to the cell cycle arrest, it has been suggested that, at least for p16/INK4, mediated growth arrest is tightly associated with the status of Rb protein phosphorylation. Therefore, it would be extremely useful to have adenoviral vectors that express p16/INK4 in order to investigate the association between Rb and p16-mediated cell cycle arrest. An additional adenoviral vector construct that would be useful in the understanding of the biochemical mechanisms that control growth regulation is an adenoviral vector that expresses the cyclin kinase p27/kip1.

Adenoviral vector constructs that would be useful in exploring the clinical utility of suicidal enzymes for the gene therapy of breast cancer is one which expresses E. coli cytosine deaminase. While adenoviral vectors have many attractive features, a key problem with adenoviral vectors is that they can only infect a small population of cancer cells within a tumor mass, leaving many of the cells uninfected. Thus, there is a need to develop adenoviral

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0 vectors which should induce cytotoxicity across the whole
tumor should adenovirus infect only a small number of tumor
cells. One approach would be to use adenoviral vectors
which under certain circumstances can be made to produce
5 cytotoxic products which are smaller in size and hence will
have opportunities to escape the cells and kill the
uninfected cells.

There are other applications in which adenoviral
vectors would prove to be extremely useful. For example,
10 during acute chemotherapy, many breast cancer patients often
acquire resistance to various drugs. Although a great deal
is known about the molecular mechanisms by which tumor cells
acquire drug resistance, there are relatively limited
approaches to treat cancers once the drug resistance has
15 been acquired. Thus, there is a need for approaches for
treating drug resistant cancers.

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SUMMARY OF THE INVENTION:

The invention provides for methods of constructing recombinant adenoviral vectors capable of expressing human cDNAs, such as wild-type p53 cDNA, called herein AdWtp53; WAF1/Cip1 cDNA, called herein AdWAF1; p27/kip1, called herein Adp27; E. coli cytosine deaminase, called herein AdCD; wild-type p16, called herein Adp16; TAM 67 (a jun/fos dominant negative mutant), called herein AdTAM67; and B7-1 and B7-2, called herein AdB7-1 and AdB7-2, respectively.

The invention further provides a method of inhibiting the growth and/or the cell cycle of proliferating cells. This method comprises contacting the cells with a recombinant adenovirus vector capable of expressing human cDNAs in an amount effective to inhibit cell proliferation.

The invention also provides a composition for contacting cells with an amount of a recombinant adenovirus vector capable of expressing human cDNAs in an amount effective to inhibit cell proliferation.

The invention additionally provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of a recombinant adenovirus vector capable of expressing human cDNAs so as to inhibit proliferation of the tumor cells.

Further, the invention provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of a recombinant adenovirus capable of expressing human cDNAs in the presence of a chemotherapeutic agent so as to inhibit proliferation of the tumor cells.

Additionally, the invention provides a method of treating a subject afflicted with a tumor which comprises contacting the tumor with an effective amount of a recombinant adenovirus capable of expressing human cDNAs in the presence of an amount of irradiation so as to inhibit proliferation of the tumor cells.

The invention also provides for the use of adenoviral

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vectors in cancer gene therapy as a mechanism for purging bone marrow cells of possible breast cancer contaminants.

The invention also provides for the use of adenoviral vectors in combination with toxins and cytotoxic drugs as a mechanism for purging bone marrow cells of possible breast cancer contaminants.

The invention further provides for the use of adenoviral vectors to eradicate cancer cells and tumors by contacting the cancer cells with an amount of adenoviral vector sufficient to eradicate the cancer cells.

Further, the invention provides for the use of adenoviral vectors as a preventative mechanism for the development of cancer in subjects who are at risk of developing cancer.

The invention also provides for a method of treating a subject afflicted with a tumor that has shown resistance to drugs which comprises contacting the tumor with an effective amount of a recombinant adenovirus capable of expressing human cDNAs so as to inhibit proliferation of the tumor cells.

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BRIEF DESCRIPTION OF THE FIGURES:

How these and other objects of this invention are achieved will become apparent in the light of the accompanying disclosure and with reference to the accompanying drawings.

Figure 1: Structure of the Recombinant Adenoviral Vector AdWtp53. A diagrammatical representation of the structure of the recombinant adenoviral vector AdWtp53 is shown. The top, hatched segment of the diagram represents the adenovirus type 5 genome, consisting approximately of 9.24-100 map units (mu). The bottom portion of the diagram represents an enlargement of the human-wild type p53 expression cassette. The human-wild type p53 expression cassette contains a left inverted terminal repeat (ITR), an origin of replication, encapsidation signals, and an Ela enhancer derived from adenovirus type 5 (stippled segments). The expression cassette also contains human wild-type p53 cDNA (solid segments) and an SV40 maturation signal (right blank segment).

Figure 2A: Immunoprecipitation of ³⁵S-labeled human wild-type p53 protein from H-358 cells exposed to varying doses of AdWtp53 or AdControl. This figure shows the immunoprecipitation of ³⁵S-labeled human wild-type p53 protein from H-358 cells exposed to increasing doses of AdWtp53 or AdControl. After labeling the cells with ³⁵S-methionine-cysteine, cell lysates were immunoprecipitated using anti-p53 antibody, solubilized protein samples loaded on 8% SDS-polyacrylamide gel electrophoresis, gels dried and exposed to X-ray film. The left panel shows radioactive signals of p53 precipitates from H-358 cells exposed to AdControl at 0.1, 1, 10 and 50 pfu/cell. The numbers 0.1 through 50 denoted above the lanes represent the pfu/cell. The arrow indicates the position of migration of p53 protein.

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Figure 2B: Immunoprecipitation of ^{35}S -labeled p53 protein from various cell lines. Cell lines, MCF-10, MCF-7, MDA-MB-231 and Adr^R MCF-7 were exposed to AdWtp53 (50 pfu/cell) or AdControl (adenovirus alone; 50 pfu/cell), and p53 protein was immunoprecipitated as set forth in Example 8. The left panel shows the results of p53 immunoprecipitation of uninfected cells, the middle panel shows the immunoprecipitation of cell exposed to AdControl, and the right panel indicates the results of immunoprecipitation of cells exposed to AdWtp53. The arrow indicates the position of migration of p53 protein.

Figure 3A: Effect of AdWtp53 and AdControl on H-358 cell growth. 5×10^4 cells were plated in triplicate on 6 well tissue culture plates, exposed to AdWtp53 (10 pfu/cells) or AdControl (10 pfu/cell), and the cell number counted on each day. Shown are cell number of H-358 cells: uninfected (●), exposed to AdWtp53 (■), and exposed to AdControl (■). Values shown are mean \pm SE.

Figure 3B: Effect of AdWtp53 and AdControl on MDA-MB-231 cell growth. 5×10^4 cells were plated in triplicate on 6 well tissue culture plates, exposed to AdWtp53 (10 pfu/cells) or AdControl (10 pfu/cell), and the cell number counted on each day. Shown are cell number of MDA-MB-231 cells: uninfected (●), exposed to AdWtp53 (■), and exposed to AdControl (■). Values shown are mean \pm SE.

Figure 3C: Effect of AdWtp53 and AdControl on MCF-7 cell growth. 5×10^4 cells were plated in triplicate on 6 well tissue culture plates, exposed to AdWtp53 (10 pfu/cells) or AdControl (10 pfu/cell), and the cell number counted on each day. Shown are cell number of MCF-7 cells: uninfected (), exposed to AdWtp53 (■) exposed to AdControl (■). Values

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shown are mean \pm SE.

Figure 4: Effect of AdWtp53 on the survival rate of different cells. Cells were exposed to different concentrations of AdWtp53 for 7 days and the survival rates calculated by the colorimetric method described in Example 3. Shown are the percent survival values for each cell line using different pfu/cell, as shown in Figure 3. The results for each cell line are represented by the following symbols: MDA-MB-157 (■), H-358 (◆), MDA-MB-231 (●), MDA-MB-453 (▲), MCF-7 (●), MCF-10 (■), 184B5 (▲), NMECs (◆), and represent the mean of triplicate determinations.

Figure 5: Western blot analysis of p53, WAF1/Cip1, mdm2 and actin proteins in breast cancer cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-453, MCF-7), a lung cancer cell line (H-358), immortalized mammary cells (MCF10, 184B5) and NMECs. Cells at a concentration of 0.5×10^6 were plated in 6 cm tissue culture dishes and infected with either 10 or 50 pfu/cell of AdWtp53 or 50 pfu/cell of AdControl for 24 hours. The cells were harvested and resuspended in 1 ml of 1xSDS-polyacrylamide gel electrophoresis buffer and 15 μ g of protein were separated in a 8% SDS polyacrylamide gel, electroblotted onto nitrocellulose, and the membranes reacted with antibodies corresponding to p53, WAF1/Cip1, mdm2 and actin. Protein bands were detected by autoradiography of X-ray film. The type of each cell line used is shown on the top of the panel. Numbers 10 or 50 on top of the lanes represent the amount of AdControl or AdWtp53 (pfu/cell). The antibodies used for detecting proteins are indicated on the left side of the panel. Protein molecular weight markers are indicated on the right side of the panel.

Figure 6: Northern blot analysis of p53 mRNA in cells

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exposed to AdWtp53. Cells (2×10^6) were plated and 24 hours later infected with AdWtp53 or AdControl (10 pfu/cell). 24 hours after infection, RNA was prepared and subjected to Northern blot analysis as described in Example 6. After transferring RNA to Magna NT membranes, blots were either probed with a p53 or a 36B4 cDNA probe. The results of autoradiograms obtained from different cells are shown on top of the lane, exposed to either AdControl or AdWtp53 as shown.

Figure 7: Nucleosomal DNA fragmentation in AdWtp53-infected MDA-MB-231, MCF-7 and NMECs. 2×10^6 cells were plated in 10 cm dishes and exposed to either AdControl or AdWtp53. 1 day after infection, the cells were collected, incubated with a lysis buffer. Low molecular weight DNA was then prepared and subjected to an agarose gel electrophoresis. The results shown are the DNA pattern observed in various cell lines (shown on top of the lane) infected with 50 pfu/cell of either AdControl or AdWtp53. The numbers on the left side of the panel indicate the position of molecular weight markers (bp).

Figure 8: Construction of the recombinant adenoviral vector AdWAF1. A diagrammatical representation of the method of construction of the adenoviral vectors AdWtp53 and AdWAF1 is shown. The Clal restriction site which was added by the invention is the upstream site, located within the first 900 nucleotides from the 5' end of the adenovirus genome.

Figure 9: Structure of the recombinant adenoviral vectors AdWtp53 and AdWAF1. This Figure represents the structure of the recombinant adenoviral vectors AdWtp53 and AdWAF1. On the top, the hatched segment represents adenovirus type 5 genome of 9.24 mu-100 mu. On the bottom is shown the enlargement of human wild type p53 and WAF1/Cip1 expression cassette. The expression cassettes contains left inverted

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terminal repeat (ITR), origin of replication, encapsidation signals and Ela enhancer derived from adenovirus type 5 (stippled segments); human cytomegalovirus immediate early promotor (left blank segment); human wild type p53 or WAF1/Cip1 cDNA (solid segment) and SV40 RNA maturation signal (right blank segment). AdWAF1 has the identical genomic structure as AdWtp53 except p53 cDNA is replaced by WAF1/Cip1 cDNA.

Figure 10: Western blot analysis of p53, WAF1/Cip1 and actin proteins in various cell lines following AdWtp53 and AdWAF1 infection. This Figure sets forth a Western blot analysis of p53, WAF1/Cip1 and actin proteins in various cell lines following AdWtp53 and AdWAF1 infection. The cells were infected with 50 pfu/cell of AdControl, AdWtp53 or AdWAF1 for 48 hours and subjected to Western blot analysis. The cell lines used are indicated on the top of the panel and the antibodies on the left side of the panel. The protein molecular weight markers are indicated on the right side of the panel.

Figures 11A-C: Effect of AdWtp53 and AdControl on cell growth. These Figures set forth the effect of AdWtp53 and AdControl on cell growth. The cells were exposed to AdWtp53 (10 pfu/cell), AdWAF1 (10 pfu/cell), and AdControl (10 pfu/cell), and the cell number was counted on each day. Shown are the cell number of H-358 cells (Figure 11A), MDA-MB-231 cells (Figure 11B) and MCF-7 cells (Figure 11C). The cell number in AdWtp53 infected cells are shown by (■), AdWAF1 infected cells by (●), AdControl infected cells by (□) and uninfected cells by (○). The values shown are mean \pm SE.

Figure 12A-D: Cell cycle analysis of MDA-MB-231 cells infected with AdWtp53 and AdWAF1. These Figures set forth

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a cell cycle analysis of MDA-MB-231 cells infected with AdWtp53 and AdWAF1. MDA-MB-231 cells were infected with AdControl, AdWtp53 or AdWAF1 (50 pfu/cell) and 24 hours later were subjected to cell cycle analysis as described in Example 13. The results shown are the cell cycle analysis of uninfected cells (Figure 12A); AdControl infected cells (Figure 12B); AdWtp53 infected cells (Figure 12C); and AdWAF1 infected cells (Figure 12D).

Figures 13A-D: Percentage distribution of cells in different cell cycle stages following AdWtp53 and AdWAF1 infection. These Figures set forth the percentage distribution of cells in different cell cycle stages following AdWtp53 and AdWAF1 infection. The cells were exposed to AdWtp53, AdWAF1 or AdControl and subjected to cell cycle analysis as described in Example 16. Shown are the percentage of cells in G1 (solid bars), S (hatched bars) and G2 + M (dashed bars) in MDA-MB-231 cells (Figure 13A); H-358 cells (Figure 13B); MCF-7 cells (Figure 13C); and NMECs (Figure 13D).

Figures 14A-E: Detection of Apoptosis in AdWtp53 and AdWAF1-infected Cells. These Figures set forth the detection of apoptosis in AdWtp53 and AdWAF1-infected cells. Two days after infection, adherent and floating cells were collected and incubated with a lysis buffer. Low molecular weight DNA was then prepared and analyzed by agarose gel electrophoresis. The results shown (Figures 14A-C) are the DNA pattern observed in various cell lines (as indicated on top of the lane) infected with 50 pfu/cell of either AdControl, AdWtp53 or AdWAF1. The positions of the molecular weight markers (bp) are indicated on the left side of the panel. In parallel experiments, MDA-MB-231 cells were also subjected to cell cycle analysis. Shown are the results following infection with AdWtp53 (Figure 14D) or AdWAF1 (Figure 14E) infection (10 pfu/cell, 24 hour

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infection). The arrow in Figure 14D indicates the position of apoptotic fragmented cells.

Figure 15: Ad.RSV β gal expression in MDA-MB-231 and human bone marrow cells. This Figure sets forth Ad.RSV β gal expression in MDA-MB-231 and human bone marrow cells. The cells (2×10^4) were infected with different moi of Ad.RSV β gal (0-10,000 pfu/cell) and β -gal activity determined as described in Example 7. The results show the β -gal activity obtained in MDA-MB-231 (●) and human bone marrow cells (o). The results indicate the average of the triplicate determinations.

Figure 16: Effect of AdWTp53 on the MDA-MB-231 and human bone marrow cell's viability. This Figure indicates the effect of AdWTp53 on the MDA-MB-231 and human bone marrow cell's viability. Freshly trypsinized MDA-MB-231 cells were mixed with CD34⁺ bone marrow cells and infected with different pfu/cell of AdWTp53. The cells were then assayed for colonogenicity as described in Example 19. The results shown are the percentage number of colonies formed after each treatment, assuming uninfected cells to be 100 %. The results of MDA-MB-231 cells are shown by (●) and of CD34⁺ cells by (o) and are the average of the triplicate determinations.

Figure 17: Ad-mediated transfection of CMV β -gal plasmid in MDA-MB-231 and human bone marrow cells. Cells (2×10^5) were transfected using CMV β -gal plasmid and different moi of dl312. After a 24 hour incubation at 37° C, the cells were lysed and β -gal activity measured. β -gal activity obtained in MDA-MB-231 cells is shown by (●) and in bone marrow cells by (o). The results are of the mean of the triplicate determinations.

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Figure 18: Ad-mediated enhancement in the cytotoxicity of a plasmid pULI100 in breast tumor and bone marrow cells. MDA-MB-231 (500), and CD34+ bone marrow cells (1000) were transfected with pULI100 plasmid in the absence and presence of dl312 (10 pfu/cell) and lipofectamine. Cell survival was estimated for MDA-MB-231 by a calorimetric assay and for CD34+ bone marrow cells by the colony forming assay described in Example 15. Uninfected cells were treated as 100% survival. Results of MDA-MB-231 cells are shown by solid bars, and bone marrow cells by the hatched bars. Results shown are the average of the triplicate determinations.

Figures 19A and 19B: AdWtp53-induced apoptosis in human breast cancer cells. Shown are cells infected with AdControl (Figure 19A) and with AdWtp53 (Figure 19B).

Figure 20: Effect of AdWtp53 injection on the growth of MDA-MB-231 xenografts in nude mice. MDA-MB-231 cells were injected subcutaneously in nude mice. 2 weeks after injection (day 0) tumors were given weekly injections of either AdControl (10⁹ pfus) or AdWtp53 (10⁹ pfus). Tumor sizes were measured on the days shown in Figure 20 and are represented by a solid bar for AdControl and a hatched bar for AdWtp53 infected tumors.

Figures 21A and 21B: Nude Mice Photos. Figure 19A shows a photograph of the animal that received an injection of AdWtp53, indicating that the tumor size disappeared completely. Figure 19B shows a photograph of the animal that received an injection of AdControl, indicating that the tumor size increased further.

Figure 22: β -gal Expression in MDA-MB-231 and Human Bone Marrow Cells Following Transfection with a Plasmid CMV β -gal in the Absence and Presence of dl312 and lipofectamine. The

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dotted bars represent the enzyme activity obtained in MDA-MB-231 cells and solid bars in CD34+ cells from G-CSF-mobilized human peripheral blood cells. Results shown are the mean of the triplicate determinations \pm S.D.

Figure 23: Cell Cycle Analysis of Human Aortic Vascular Smooth Muscle Cells Infected with AdWtp53 and AdWAF1. 2×10^5 of human aorta vascular smooth muscle cells were infected with AdWtp53, AdWAF1 and AdControl (50 pfu/cell) for 48 hours and subjected to cell cycle analysis. Results shown are the cell cycle analysis of uninfected cells (Panel A), AdControl-infected cells (Panel B), AdWAF1-infected cells (Panel C) and AdWtp53-infected cells (Panel D). The arrow indicates the population of cells in G1 subgroup. Changes in the percentage of cells exposed to various doses of (1, 10 and 50 pfu/cell) AdWtp53 (■) and AdWAF1 (●) in G1 phase (Panel E), S phase (Panel F) and G2/M phase (Panel G) are shown.

Figure 24: Cytotoxicity of AdWtp53, AdWAF1 and AdControl to human aortic vascular smooth muscle cells. The cytotoxicity of each adenovirus on human aortic vascular smooth muscle cells (250/well) was determined in triplicate on each wells of 96 well plates, exposed to AdWtp53 (■), AdWAF1 (●) and AdControl (□) of up to 40000 pfu/cell and after 7 days the number of cells was analyzed by colorimetric assay. Values shown are mean \pm S.E.

Figure 25: Figure 25 sets forth a schematic diagram of Adp27.

Figure 26: Figure 26 shows Adp27-mediated p27 Expression in Human Breast Cancer Cells.

Figures 27A and 27B: Effect of Adp27-mediated expression of p27 on DNA Cell Cycle Histograms.

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Figure 28: Effect of Adp27 Infection on Apoptosis.

Figure 29: Figure 29 sets forth a schematic diagram of AdCD.

Figures 30A and 30B: Figure 30A sets forth AdCD-mediated cytotoxicity of MCF-7 cells in the presence of 5-FC. Figure 30B sets forth AdCD-mediated cytotoxicity of MDA-MD-231 in the presence of 5-FC.

Figure 31: Figure 31 sets forth a schematic diagram of Adp16.

Figure 32: Figure 32 shows Adp16-mediated p16 expression in various cell cancer lines.

Figure 33: Figure 33 sets forth a schematic diagram of AdTAM67.

Figure 34: Figure 34 sets forth a schematic diagram of AdB7-1.

Figure 35: Figure 35 sets forth a schematic diagram of AdB7-2.

Figure 36: Figure 36 depicts AdWtp53-mediated Expression.

Figure 37: Figure 37 illustrates the effect of AdWtp53 Infection on Cell Cycle.

Figure 38: Figure 38 sets forth the Effects of AdWtp53 on Apoptosis.

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DETAILED DESCRIPTION OF THE INVENTION:

The present invention provides novel methods for the construction of recombinant adenoviral vectors capable of expressing human cDNAs. Specific non-limiting examples of such human cDNAs are wild-type p53, WAF1/Cip1/p21, p27/kip1, E. coli cytosine deaminase, wild-type p16, TAM 67 (a jun/fos dominant negative mutant) and B7-1 and B7-2.

The present invention affords a novel method for the construction of the adenoviral vectors by the addition of a second ClaI site for utilization in excising the 5' end of the adenoviral genome.

The present invention additionally provides a method of inhibiting the proliferation of cells. This method comprises contacting the cells with an adenoviral vector, capable of expressing human cDNAs, in an amount effective to inhibit cell proliferation. Particular non-limiting examples of such vectors are AdWTP53, AdWAF1, Adp27, AdCD, Adp16, AdTAM67, AdB7-1 and AdB7-2.

Further, the invention provides a method of inhibiting the cell cycle of proliferating cells. This method comprises contacting the cells with an adenoviral vector, capable of expressing human cDNAs, in an amount effective to inhibit DNA synthesis.

The present invention also provides a method of eradicating cancer cells by contacting the cells with an adenoviral vector, capable of expressing human cDNAs, in an amount effective to eradicate the cancer cells.

The recombinant adenoviral vector as described herein can be used and engineered to contain and express other genes (i.e. cDNAs) that may be useful for eradicating tumor cells in which the vector is expressed via the toxic effects of the expressed genes. Non-limiting examples of other cDNAs that can be used in the adenovirus vectors of the invention are HSVTK, No-synthetase, GADD 45, p15, mdm2, Rb, BAX, IL2, GMCF, p53-antisense, Her/Neu2 antisense, and Erb4 antisense.

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0 The present invention also provides for a method of
treating a subject afflicted with a tumor that has shown
resistance to drugs which comprises contacting the tumor
with an effective amount of a recombinant adenovirus capable
of expressing human cDNAs so as to inhibit proliferation of
5 the tumor cells.

A preferred recombinant adenovirus vector expressing
human wild-type p53 cDNA is AdWtp53. AdWtp53 possesses a
human-wild type p53 expression cassette consisting of a left
10 inverted terminal repeat, an origin of replication,
encapsidation signals, an E1a enhancer derived from
adenovirus type 5, a 1.7 kb human wild-type p53 cDNA, and an
SV40 maturation signal.

The amount of the recombinant adenovirus vector
expressing human wild-type cDNA effective to inhibit cell
15 proliferation of actively proliferating cells will vary
according to the cell type. Maximal inhibition of cell
proliferation by the recombinant adenovirus vector is
achieved on cancer cells that are either deficient in the
p53 protein or those that express a mutant p53 protein.

20 The present invention further provides a method of
treating a subject afflicted with a tumor which comprises
contacting the tumor with an amount of a recombinant
adenovirus vector capable of expressing human cDNAs which is
administered to the subject previous to, simultaneously, or
25 subsequent to, administration of a chemotherapeutic agent or
to an amount of irradiation effective to treat the tumor.
Examples of chemotherapeutic agents are known to those
skilled in the art and include, but are not limited to,
bleomycin, mitomycin, cyclophosphamide, doxorubicin,
30 paclitaxel, and cisplatin (See El-Deiry, et al., Cell,
75:817-825 (1993)).

In one embodiment of the invention, the recombinant
adenovirus vector containing a gene or cDNA of interest is
administered in a pharmaceutically acceptable carrier. A
35 pharmaceutically acceptable carrier encompasses any of the

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standard pharmaceutical carriers such as sterile solution, tablets, coated tablets and capsules. Such carriers may typically contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stensic acid, talc, vegetable fats or olis, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives and other ingredients.

The administration of the composition may be effected by any of the well known methods, including but not limited to, oral, intravenous, intramuscular, and subcutaneous administration. The preferred method of administration of the composition is local, i.e. at the site of the tumor.

In the practice of the method of this invention the amount of a recombinant adenovirus vector capable of expressing human cDNAs incorporated in the composition may vary widely. Methods for determining the precise amount depend upon the subject being treated, the specific pharmaceutical carrier, the route of administration being employed, the frequency with which the compound is to be administered, and whether the composition is administered in conjunction with a chemotherapeutic agent and/or irradiation treatment. The preferred amount of the vectors which may be administered for effective inhibition of proliferation of cells ranges from 10^8 to 10^{10} pfu/tumor.

The invention provides a novel method for the construction of adenoviral vectors. This method is not hampered by the problems which presently exist in the construction of adenoviral vectors. The invention provides a construction technique whereby an additional ClaI site is introduced at the 5' end of the adenovirus genome. The introduction of the second ClaI site at the 5' end of the adenovirus genome greatly reduces the chances of obtaining undigested genome as only one of the two ClaI sites will have to be cut to prevent the production of the non-recombinant background infectious virions. Moreover, because the novel adenovirus vectors will be made using

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viral genomic DNA as the starting point, there is no need to utilize a plasmid based vector.

In one embodiment of the invention, an adenovirus vector expressing human wild-type p53 cDNA (AdWtp53) was constructed. In the AdWtp53 genome, an additional ClaI restriction site was generated, thus providing two(2) ClaI restriction sites for excising the 5' end of the adenovirus genome. AdWtp53 was used as a parental adenovirus genome to generate future recombinant adenoviruses. In a further embodiment of the invention, a recombinant adenovirus which contains WAF1/Cip1 cDNA (AdWAF1) was constructed.

Utilizing two recombinant adenoviruses expressing wild-type p53 and WAF1/Cip1 cDNAs, it was determined that p53-mediated effects on cell cycle arrest are associated with the WAF1/Cip1 expression, and WAF1/Cip1 overexpression in the absence of p53 overexpression fails to induce apoptosis. Thus, the effects of p53 on apoptosis apparently require the cooperation of other signal transduction agents besides WAF1/Cip1.

In another embodiment of the invention, an adenovirus vector expressing human WAF1/Cip1/p21 cDNA (AdWAF1) was constructed. Recently, radiation-induced p53 effects were shown to be associated with WAF1/Cip1 induction (El-Deiry, et al., Cancer Res., 54:1169-1174 (1994)). However, whether WAF1/Cip1 overexpression in the absence of other p53-mediated signals induces apoptosis was not clear. However, the construction of the adenovirus vectors AdWtp52 and AdWAF1 of the present invention, which can both induce WAF1/Cip1 gene overexpression, allowed the direct investigation of the role of WAF1/Cip1 induction independent of p53 overexpression. It appears that AdWtp53-mediated p53 overexpression induces expression of WAF1/Cip1 in cells resulting in cell cycle arrest in all cells studied and apoptosis in those cells lacking expression of endogenous p53 or cells expressing mutant p53. In contrast, AdWAF1-mediated WAF1/Cip1 expression in infected cells resulted in

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cell cycle arrest without inducing apoptosis. Thus, the cytotoxic effects of wild type p53 involves additional factors besides WAF1/Cip1. It is already known that p53 overexpression can transcriptionally activate several genes, including MDM2 (Katayose, et al., Clin. Cancer Res. (Submitted 1995); Momand, et al. Cell 69:1237-1245 (1992)) GADD45 (Smith, et al. Science 266, 1376-1379 (1994)). The p53-mediated apoptotic pathway could involve induction of these or previously unidentified genes.

It is also important to note that AdWtp53 and AdWAF1 infection produces marked differences in cell cycle arrest. Infection with AdWtp53 resulted in a decline in the S phase and an increase in the proportion of G2+M. In contrast, AdWAF1 infection caused a marked increase in the proportion of cells in the G1 phase and a decline in S phase cells. These results indicated that WAF1/Cip1 overexpression causes cell cycle arrest at G1/S checkpoint while p53 can result in cell cycle arrest at G1/S and G2+M checkpoints. While the effects of both of these vectors on G1/S arrest can be explained by the WAF1/Cip1-mediated inhibition of CDK kinases, resulting in dephosphorylation of Rb protein (Dulic, et al., Cell 76:1013-1023 (1994); Michalovitz, et al., Cell 62:671-680 (1990); Ewen, M. E., Cancer Metastasis Rev. 13:45-66 (1994)) the mechanisms of AdWtp53-mediated accumulation of G2 + M cells remain unknown. It may be speculated that the cells arrested in G2 + M stage (in response to p53 overexpression) are perhaps more prone to undergo apoptosis, while G1/S arrested cells (in response to WAF1/Cip1 induction) remained viable for at least limited time period.

The differential effects of p53 and WAF1/Cip1 overexpression on cell cycle arrest and apoptosis were translated in the overall cytotoxic effects of AdWtp53 and AdWAF1. Thus, cells (H-358 (lung cancer cell line) and MDA-MB-231 (breast cancer cell line)) which undergo both cell cycle arrest and apoptosis (in response to p53

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overexpression) were more sensitive to cytotoxic effects of AdWtp53 as compared to AdWAF1; and cells (MCF-7, NMECs) which exhibit only growth arrest (by p53 or WAF1/Cip1 overexpression) were less sensitive to the cytotoxic effects of AdWtp53 and AdWAF1. Although the endogenous p53 status appears to play a role in determining the overall cytotoxicity of AdWtp53 and AdWAF1, it is important to note that the consequences of p53 and WAF1/Cip1 overexpression could be also dependent upon the amounts of p53 and WAF1/Cip1 produced in different cells, differential stability of these proteins in different cells, differential localization of these proteins within the cell, the ability or inability of these proteins to interact with other cellular factors or other downstream signals, and the absence or presence of other effector molecules (Halдар, et al., Cancer Res. 54:2095-2097 (1994)). It is also possible that the AdWtp53-mediated effects are not necessarily at the transcriptional level but perhaps involve other post transcriptional regulation as previously suggested (Caelles, et al., Nature 370:220-223 (1994)) and could also depend upon the cell's DNA repair ability (Modrich, P., Science 266:1959-1960 (1994)).

Independent of the mechanisms of the differential cytotoxicities, both AdWtp53 and AdWAF1 have clinical utility. The AdWtp53 vector may be clinically useful in tumors expressing mutant p53, which includes many human cancers (Nigro, et al., Nature 342:705-708 (1989); Takahashi, et al., Science 246:491-494 (1989); Srivastava, et al., Nature 348:747-749 (1990); Katayose, et al., Clin. Cancer Res. (Submitted 1995); Liu, et al., Cancer Res. 54:3662-3667 (1994); Fujiwara, et al., Cancer Res. 54:2287-2291 (1994)). The AdWAF1 vector may be useful in clinical settings wherein growth arrest of cells is an effective treatment.

The present invention also provides for the use of the novel AdWtp53 vector in order to elucidate the role of p53

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in inducing growth inhibition and apoptosis in vascular smooth muscle cells. Specifically, the role of p53 was analyzed by observing the effects of AdWtp53 on the proliferation and apoptosis of cultured human aortic vascular smooth muscle cells (AoVSMC) (See Example 25). In addition, the AdWAF1 vector of the present invention, expressing p53-inducible p21 cyclin-dependent kinase inhibitor, was also used to further elucidate the role of p53 in cancer.

The results of these studies have shown that adenovirus vector expressing p53 induced marked cytotoxicity in primary cultured AoVSMC. This cytotoxicity was associated with cell cycle arrest in G1 and G2/M boundary, accumulation of cells in G1 subgroup and perhaps apoptosis. Examining the mechanisms of p53-mediated cytotoxicity to AoVSCM is an important issue because as to date, there is no report to indicate that p53 induces apoptosis in normal cells, including vascular smooth muscle cells. This examination is made possible by the use of the vectors of the present invention. In this context, the involvement of p21 was studied by examining the overexpression of p21 in AoVSMC infected with either AdWtp53 or AdWAF1. Since AdWaf1 was about 200 times less toxic than AdWtp53, it is therefore unlikely that induction of p21 due to p53 overexpression mediated the cytotoxicity of AdWtp53 to AoVSMC. Further, the data herein shows that AdWtp53 induced not only G1 arrest but also G2/M arrest in AoVSMC, whereas AdWAF1 only induced G1 arrest.

The present invention further provides for the use of adenoviral vectors in cancer gene therapy. The presence of mutated p53 is widespread in different human cancers. Thus, reconstituting tumor suppressor p53 gene expression by adenoviral vectors is an attractive strategy for gene therapy. Since the adenovirus enters human epithelial cells with an efficient low-pH endosomes mediated endocytosis (Seth, et al., (1986) Pathway of adenovirus entry into

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cells, p. 191-195 In Colwell, R. and Lonberg-Holm, K. (ed.), Virus attachment and entry into cells, American Society for Microbiology, Washington, D.C.; Rosenfeld, et al., Hum. Gene Ther., 5:331-342 (1994)), tumors of mammary epithelial origin will be especially amenable to treatment by AdWTp53.

The present invention demonstrates that normal mammary epithelial cells are resistant to apoptosis by AdWTp53, while tumor cells null for p53, or expressing mutant p53, readily undergo apoptosis. These results demonstrate a specificity to AdWTp53-mediated eradication of tumor cells, lending further support for the utilization of adenoviral vectors in gene therapy.

Human adenoviruses have been used previously in gene transfer techniques in eucaryotic cells either by infecting the cells with a recombinant adenovirus expressing the transgene, or by adenovirus-mediated transfection of the plasmid DNAs.

The present invention shows that breast tumor cells are a much better target for Ad-mediated gene transfer as compared to bone marrow cells. The present invention also indicates that adenoviruses, in combination with appropriate toxic genes, can kill breast tumor cells while sparing bone marrow cells.

The present invention allows a comparison of the infectability using a replication-deficient adenovirus expressing β -galactosidase (β -gal gene). In addition, the invention can be used to determine or measure the relative cytotoxicity of the adenoviral vector encoding the catalytic domain of the Pseudomonas exotoxin gene.

The vectors of the invention cause an increase in the infection and transfection efficiencies of plasmid DNA in the presence of adenovirus in human breast tumor and bone marrow cells. Following infection of breast tumor cells with an adenovirus expressing β -galactosidase gene, high levels of β -galactosidase activity were observed, while normal bone marrow cells expressed about 500-fold less β -

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galactosidase activity. A recombinant adenovirus expressing human wild-type p53 protein (AdWTp53) was highly cytotoxic to breast tumor cells (100% of cell killing observed by 100 pfu/cell); while bone marrow cells were relatively resistant to the cytotoxic effects of AdWTp53 (less than 10% cell killing observed by 1000 pfu/cell).

One main reason that adenoviruses are effective for delivering genes to breast tumor cells as compared to bone marrow cells may relate to the high number of adenovirus receptors present on the surface of the cell. Therefore, other tumors found to have high levels of adenoviral receptors could be used in accordance with the invention. Human breast tumor cells tested were found to have fairly high number of adenovirus receptors (in the range of $5-10 \times 10^3$) while bone marrow cells are relatively deficient in cell surface adenovirus receptors (less than 5×10^2 /cell). Because adenovirus-mediated gene transfer requires the receptor-mediated uptake of adenovirus into the cells, breast cancer cells expressing adenoviral receptors would be a better target for adenoviruses than the adenovirus receptor deficient bone marrow cells.

In addition, the efficiency of adenoviral-mediated transfections may be enhanced by using a replication-deficient adenovirus mutant dl312, and even further enhanced by the addition of a liposome, such as lipofectamine and other mono and polycationic lipids. The present disclosure indicates that human bone marrow cells treated under the same conditions expressed very low levels of the transfected β -galactosidase DNA. It was also determined that transfection of cells with plasmid DNA expressing Pseudomonas exotoxin gene in the presence of dl312 and lipofectamine resulted in greater than 90% breast tumor cell killing, while human bone marrow CD34+ cells were at least 500-times more resistant to this treatment.

Since infection of breast tumor cells with a recombinant adenovirus expressing a toxic transgene (e.g., human WT p53)

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or transfecting with a toxin gene (Pseudomonas exotoxin) and in the presence of adenovirus resulted in marked cytotoxicity in breast tumor cells, with little or no toxicity to bone marrow cells, human adenoviruses are useful for cancer gene therapy and for removing or "purging" cancer cells from bone marrow. Human adenoviral vectors are also useful in cancer gene therapy for the treatment of lung, prostate, and liver cancer cells, and may be useful in the treatment of leukemia.

Adenovirus vectors which express a toxic gene may be used in gene therapy, or, alternatively, a replication-deficient adenovirus in combination with plasmid DNAs coding for a toxin gene may be used. The present disclosure indicates that since the use of both results in at least 100-fold increase in cytotoxicity to breast cancer cells compared to bone marrow cells, this approach offers a fairly wide efficacy window for purging. The protocols for human bone marrow purging using recombinant adenovirus vectors appear to be simple and effective. Recombinant adenoviruses for use in the protocols are replication-deficient, which pose few problems to the bone marrow cells. Moreover, if adenoviruses are used in combination with toxin genes, much lower dosage of adenoviruses will be required, thus making the bone marrow purging protocol even more safe. Because breast cancer cell contamination in human bone marrow which is to be used for marrow transplantation is potentially a serious problem, the adenoviral vectors of the present invention are necessary and useful tools for purging bone marrow cells of such contaminating tumor cells.

Adenoviral vectors may also be employed in the eradication of cancer cells. The effects of Adp53 on human breast tumors (derived from MDA-MB-231 cells) grown as xenografts in nude mice was investigated. MDA-MB-231 cells were injected subcutaneously in nude mice and two weeks later when the tumors were palpable, they were injected with AdWtp53 (10^9 pfus) or a control adenovirus (AdControl) (10^9

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pfus). Tumors which received a control virus continued to grow over the next 14-21 days, while tumors which received AdWTp53 exhibited inhibition of subsequent tumor growth. Tumors of nude mice that were injected with AdWTp53 disappeared completely over the course of the treatment (See Figure 21A), while the tumors of the nude mice that were injected with AdControl (adenovirus only) increased in size (See Figure 21B). These results thus confirm that the adenoviral vectors of the present invention would be extremely useful in cancer gene therapy. A preferred mode of administration is by direct injection. Therefore, the adenoviral vectors of the invention will be useful for the eradication of cancer cells by contacting the cancer cells of the tumor with an amount of the adenoviral vector sufficient for the eradication of the cancer cells. A particular example of such a vector for use in this treatment is AdWTp53. The adenoviral vector may also be administered to a subject previous to, simultaneously, or subsequent to, the administration of a chemotherapeutic agent or an amount of irradiation effective to eradicate the cancer cells.

The adenoviral vectors are also useful in the prevention of the development of cancer cells in those subjects who are at risk of developing cancer. The preventative treatment involves the administration of an adenoviral vector expressing the desired DNA which eradicates the cancer, via the toxic effects of the expressed genes, in an amount effective to prevent or inhibit the development of cancerous cells.

The present invention also provides for the construction and use of an adenoviral vector capable of expressing p16/INK4 kinase inhibitor. (See Example 5) It has been suggested that, for p16/INK4, mediated growth arrest is tightly associated with the status of Rb protein phosphorylation. In order to test the idea how p16 and Rb proteins mediated cell cycle arrest can be controlled by the

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endogenous background of p16 and Rb, the effects of adenoviral-mediated expression of p16 and Rb protein in several cell lines with different intrinsic status of p16 and Rb protein were herein investigated. (See Example 17)

As shown in Figure 32, in each cell line infection by Adp16 led to an increased expression of p16 protein. However, in cells infected with a control adenovirus, no increase in the basal level of p16 was observed.

Similarly when cells were infected with AdRb, high level protein expression of Rb protein was observed in each cell line. However, the expression of a control protein Actin was not altered following any adenoviral infection. These results indicate that Adp16-mediated expression of p16, and AdRb-mediated expression of Rb protein was independent of the endogenous status of either p16 or Rb.

The effects of p16 and Rb expression on cell cycle progression was investigated in cells with different endogenous p16 and Rb status. Different cells were infected with different doses (1-200 pfu/cell) of either Adp16, AdRb or AdControl, and 24 hours later analyzed for cell cycle distribution. Following Adp16 infection, in each cell line expressing endogenous wild type Rb protein (MCF-7, MDA-MB-231), an increase in cells in G1 and a decrease in cell number in S phase was observed, indicating that the cells were arrested at G1/S. The accumulation of cells in G1 phase was dependent upon the concentration of Adp16 used. However, in cells expressing non-functional form of Rb protein, Adp16 failed to induce cell cycle arrest. The effect of Adp16 on cell cycle arrest was not dependent on the endogenous status of p16. In each cell line used in this study (except those defective for Rb), Adp16 induced a strong cell cycle arrest, which was dependent on the dose of Adp16 used.

The present invention also provides for the construction and use of an adenoviral vector capable of expressing p27/kip1, a cell cycle inhibitor that is involved in the

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0 signal transducing effects of TGF β . (See Example 3) The
recombinant adenoviral vector Adp27 of the present invention
was used to study the relationship between p27/kip1 effects
on cell growth, cell cycle, cyclin kinases and apoptosis.
5 It is herein shown that p27/kip1 expression can regulate the
cell cycle at both G1/S and G2/M check points, and that
these effects are associated with the inhibition of cdk2
kinase and cdc2 cyclin B1-associated kinases. However,
inhibition of these kinases did not result in cellular
10 apoptosis, indicating the dissociation of cell cycle arrest
at G1/S and G2/M phase from apoptosis. Thus, while cell
cycle progression can be linked with cyclin kinase
activities, its relationship with apoptosis is much more
complex.

15 The present invention further provides for adenoviral
vectors for use in treating drug resistant cancers. The
cytotoxic effects of AdWtp53 in two drug resistant breast
cancer cells; adriamycin resistant human breast cancer MCF-7
cells (MCFV-Adr) and mitoxantrone resistant MCF-7 cells,
were herein investigated. (See Example 25) Following
20 infection by AdWtp53, all the cell lines expressed high
levels of p53 protein. However, MCF-Adr and MCF-Mito cells
were much more sensitive to killing by AdWtp53 as compared
to the parental MCF-7 cells. (See Table 5) The AdWtp53-
mediated cytotoxicity in drug resistant cells was associated
25 with cell cycle arrest (in G1/S and G2/M phase); inhibition
of at least two cyclin kinase cdk2 and cdc2 cyclin B1-
associated kinases, and apoptosis. Thus, the use of the
novel adenoviral vectors of the present satisfies a needed
requirement for approaches for treatment of drug resistant
30 cancers.

The present invention further provides for adenoviral
vector constructs that would be useful in exploring the
clinical utility of suicidal enzymes for the gene therapy of
breast cancer. While adenoviral vectors have many
35 attractive features, a key problem with adenoviral vectors

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is that they can only infect a small population of cancer cells within a tumor mass, leaving many of the cells uninfected. Thus, there is a need to develop adenoviral vectors which should induce cytotoxicity across the whole tumor should adenovirus infect only a small number of tumor cells. One approach would be to use adenoviral vectors which under certain circumstances can be made to produce cytotoxic products which are smaller in size and hence will have opportunities to escape the cells and kill the uninfected cells; or diffuse across cell boundaries (e.g. through gap junctions) providing bystander effect. One such gene is E. coli deaminase which can convert a pro-drug 5-fluoro cytosine into a toxic species 5-fluoro uracil. Thus, the present invention provides for the construction and use of an adenoviral vector capable of expressing E. coli cytosine deaminase. (See Example 4) It is shown herein that the presence of 5-FC, AdCD is extremely cytotoxic to MDA-MB-231 and MCF-7 breast cancer cells, and show that bystander effects play a role in overall cytotoxicity of AdCD. Further, investigation of the cytotoxic effects of AdCD in vivo in the presence of 5-FC in human breast tumors grown as xenografts in nude mice. Thus, AdCD has potential clinical applications for the treatment of breast cancer. Because adenoviruses can express the transgenes to very high levels, coupled with the bystander effects of the suicidal genes, AdCD has advantages over retroviruses expressing E. coli CD.

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EXAMPLES

The Examples herein are meant to exemplify the various aspects of carrying out the invention and are not intended to limit the scope of the invention in any way. The Examples do not include detailed descriptions for conventional methods employed in the construction of vectors, the insertion of cDNA into such vectors, or the introduction of the resulting vectors into the appropriate host. Such methods are well known to those skilled in the art and are described in numerous publications, including Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA, (1989).

EXAMPLE 1**Construction of an Adenovirus Vector Coding For the Expression of Human Wild-Type p53 Protein (AdWtp53).**

Homologous recombination between a shuttle vector pDK10, containing an expression cassette of human wild type p53 cDNA and the adenovirus genome cloned in a plasmid pJM17 generated an adenovirus clone in which the adenovirus E1 region was replaced by the wild type p53 cDNA expression cassette.

Plasmid AdWtp53 was constructed using co-transfection of shuttle vector pDK10 containing the wild type p53 expression cassette and a plasmid pJM17 containing the adenovirus type 5 genome. pJM17 may be obtained from Microbix Biosystems, Inc., Toronto, Ontario Canada. pDK10 was deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland on February 17, 1995 under ATCC Accession No. 97064.

The shuttle vector pDK10 was constructed by inserting the cytomegalovirus (CMV) immediate early promotor and enhancer, a 1.7-kilobase XbaI fragment of human p53 cDNA (See Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)), the SV40 small T intron, and an SV40 polyadenylation signal into

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the ClaI site of plasmid pXCX2 (See Graham, et al. (1991) Manipulation of adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene transfer and expression protocols, Humana Press, Clifton, New Jersey).

Figure 1 shows the schematic diagram of AdWtp53. The 5' end of the genome contains the AdWtp53 expression cassette (10.3 mu) followed by the rest of the adenovirus genome. The key elements of the expression cassette of AdWtp53 include the left inverted terminal repeat (ITR), the adenoviral origin of replication, encapsidation signal, the Ela enhancer, the CMV immediate early promoter, the human wild type p53 cDNA and SV40 polyadenylation signal.

Plasmid pDK10 was co-transfected with pJM17 (See McGrory, et al., Virology, 163:614-617 (1988)) (kindly provided by F. Graham, McMaster Univ., Hamilton, Ontario) into the transformed human embryonic kidney cell line 293 (ATCC Accession No. CRL1573) by calcium phosphate mediated gene transfer technique (See Graham, et al. (1991) Manipulation of adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene transfer and expression protocols, Humana Press, Clifton, New Jersey; and Gilardi, et al., FEBS Lett., 267:60-62 (1990) (Gibco BRL, Gaithersburg, MD)). The day following transfection, the medium was replaced with a 1xMEM (Gibco BRL) containing 1% sea plaque agarose gel (FMC, Rockland, ME) and 10% fetal bovine serum (FBS) (Gibco BRL) and the cells were incubated at 37°C. Every five days 2 ml of MEM containing 1% sea plaque agarose gel and 10% FBS was added to the top of the cells until plaques were observed. Isolated plaques were picked and subjected to another cycle of infection in 293 cells as described previously (See Graham, et al. (1991) Manipulation of adenovirus vectors, p. 109-128 In Murray, E. J. (ed.), Gene transfer and expression protocols, Humana Press, Clifton, New Jersey; and Gilardi, et al., FEBS Lett., 267:60-62 (1990)).

Purified recombinant AdWtp53 was assayed for the absence of Ela and the presence of p53 sequences using the

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polymerase chain reaction (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). Briefly, 24 hours following infection with adenoviral vector, cell lysates were prepared using guanidine thiocyanate solution, and aliquots were obtained for use in the PCR analysis. In order to determine the absence of Ela nucleotide sequences, the following primers were used:

5'-TCTTGAGTGCCAGCGAGTAG-3' (SEQ ID NO:1); and

5'-CAAGGTTTGGCATAGAAACC-3' (SEQ ID NO:2).

In order to determine the presence of p53 nucleotide sequences, the following primer was selected from exon seven of the p53 nucleotide sequence:

5'-GTTGGCTCTGACTGTACC-3' (SEQ ID NO:3)

and the following downstream primer

5'-GTTCCGTCCCAGTAGATTACC-3' (SEQ ID NO:4)

was selected from exon eight of the p53 nucleotide sequence.

This combination of primers allows for the differentiation of the PCR product of the endogenous genomic p53 gene from the viral-associated p53 gene. AdWtp53 was propagated in 293 cells grown in monolayers, purified by two cesium chloride density gradients, dialyzed against a buffer containing 10% glycerol, 1 mM MgCl₂, pH 7.5, and stored at -70°C as described by Seth, et al., J. Virol. 68:933-940 (1994). PCR analysis of the purified AdWtp53 indicated that it contained p53 cDNA but was devoid of Ela sequences.

Control adenovirus vectors used in this study were: Ad.RSVβgal, an adenovirus vector containing β-galactosidase gene (Stratford-Perricaudet, et al., J. Clin. Invest., 90:

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626-630 (1992)), and AdControl, a null adenovirus vector dl312 (Jones, et al., Cell, 17:683-689 (1979) (kindly provided by R. Crystal, NIH, Bethesda)

EXAMPLE 2

Construction of an Adenovirus Vector Coding For the Expression of Human WAF1/Cip1 Protein (AdWAF1).

AdWAF1 was constructed by homologous recombination between a shuttle vector containing WAF1 (p21 cyclin-dependent kinase inhibitor) cDNA expression cassette (pDK13, see Figure 8) and Clal cut genomic DNA derived from AdWTP53 using a previously described procedure (See Graham, F. L. and Prevec, L. (1991) *In Gene transfer and expression protocols*, (Murray, E. J. ed.) pp. 109-128, Humana Press, Clifton, New Jersey) to purify adenovirus genomic DNA. pDK13 was deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland on February 17, 1995 under ATCC Accession No. 97063. Following co-transfection into the transformed human embryonic kidney cell line 293 (obtained from American Type Tissue Culture Collection under ATCC Accession No. CRL 1573), adenovirus plaques were isolated as described previously (Katayose, et al., *Clin. Cancer Res.* (Submitted 1995)). The presence of WAF1 cDNA in AdWAF1 was confirmed by polymerase chain analysis using the following primers:

5'-AGTCTCAGTTTGTGTGTCTTA-3' (SEQ ID NO:5);

5'-GTGCCATCTGTTTACTTCTCA-3' (SEQ ID NO:6).

AdControl used in this study was a replication-deficient adenovirus dl312 (Jones, N. and Shenk, T., Cell, 17:683-689 (1979)) (kindly provided by T. Shenk, Princeton University, Princeton, NJ). Adenoviruses were propagated in 293 cells, purified by two cesium chloride density centrifugation, tittered and stored at -70°C as described previously

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(Katayose, et al., Clin. Cancer Res. (Submitted 1995); Seth, et al., J. Virol. 68:933-940 (1994)).

EXAMPLE 3

Construction of a Recombinant Adenoviral Vector Coding for the Expression of Human p27 Protein (Adp27).

Adp27 was constructed by co-transfection of a shuttle vector pCG1 containing the p27 expression cassette and 35 kb fragment derived from an adenovirus expressing p53 (AdWtp53). The shuttle vector pCG1 was constructed by inserting the human cytomegalovirus (CMV) immediate promoter and enhancer, a 1.7 kilobase XbI fragment of p27 cDNA into a null shuttle vector described previously. The two DNAs were cotransfected into the transformed human embryonic kidney cell line 293 (ATCC CRL1573) by calcium phosphate mediated gene transfer technique (Gibco BRL, Gaithersburg, MD). After 24 hours, the medium was replaced with a 1xMEM (Gibco BRL) containing 1% sea plaque agarose gel (FMC, Rockland, ME) and 10% fetal bovine serum (FBS) (Gibco BRL). Every five days, 2 ml of MEM containing 1% sea plaque agarose gel and 10% FBS was added to the top of the cells until plaques were observed. Isolated plaques were picked and subjected to another cycle of infection in 293 cells as described previously. Several plaques were screened for the presence of p27 sequences by polymerase chain reaction (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). PCR reactions were also performed for E1 and p53 sequences using sets of primers described previously. Figure 25 shows the schematic diagram of Adp27. The 5' end of the genome contains the Adp27 expression cassette (10.3 mu) followed by the rest of the adenoviral genome.

The key elements of the expression cassette of Adp27 include the left inverted terminal repeat (ITR), the adenoviral origin of replication, encapsidation signal, the Ela enhancer, the CMV immediate early promoter, the human

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wild-type p53 cDNA and SV40 polyadenylation signal. Adp27 has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

EXAMPLE 4

Construction of a Recombinant Adenoviral Vector Coding for the Expression of E. Coli Cytosine Deaminase (AdCD).

AdCD was constructed by co-transfection of a shuttle vector pPS1 containing the E. coli cytosine deaminase expression cassette and 35 kb fragment derived from ClaI cut adenoviral genomic DNA as previously described. Plasmid pPS1 was constructed by inserting the human cytomegalovirus (CMV) early promoter and enhancer, a 1.1 kilobase XbaI fragment of CD cDNA into a null shuttle vector. Co-transfection and isolation of viral plaques were performed by published methods. Several plaques were screened for the presence of CD sequences by polymerase chain reactions (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). PCR reactions were also performed for E1 using sets of primers described previously. Figure 29 shows the schematic diagram of AdCD. The 5' end of the genome contains the AdCD expression cassette (10.3 mu) followed by the rest of the adenoviral genome. The key elements of the expression cassette of AdCD include the left inverted terminal repeat (ITR), the adenoviral origin of replication, encapsidation signal, the E1a enhancer, the CMV immediate early promotor, E. coli CD gene and SV40 polyadenylation signal. AdCD has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

EXAMPLE 5

Construction of a Recombinant Adenoviral Vector Coding for the Expression of Human p16 (Adp16).

A recombinant adenovirus expressing human p16 (Adp16) was constructed by using homologous recombination methods

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described previously to construct other adenoviral vectors. In brief, a shuttle vector pCG2 containing the p16 expression cassette was co-transfected with a 35 kb fragment derived from ClaI cut adenoviral genomic DNA in transformed human kidney 293 cells. Adenoviral plaques were picked and subjected to another cycle of infection in 293 cells as described previously. Several plaques were screened for the presence of p16 sequences by polymerase chain reaction (PCR) according to published methods (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993)). PCR reactions were also performed for E1 as described previously. A schematic diagram of Adp16 is set forth in Figure 31. Adp16 has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

Similarly, the following adenoviral vectors were prepared as set forth above:

(1) AdTAM67 (See Figure 33 for the schematic diagram).

(2) AdB7-1 (See Figure 34 for the schematic diagram).

(3) AdB7-2 (See Figure 35 for the schematic diagram).

AdTAM67, AdB7-1 and Adb7-2 have been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland.

EXAMPLE 6

Preparation of Cell Culture

Breast cancer cell lines, MDA-MB-231 (ATCC Accession No. HTB26), MCF-7 (kindly provided by R. Buick, Univ. of Toronto), Adr^R MCF-7, adriamycin resistant MCF-7 cells and MCF-Mito, mitoxantrone resistant MCF-7 cells (Fairchild, et al., Cancer Res., 47:5141-5148 (1987)) were cultured in alpha minimal essential medium (MEM) (Gibco BRL) supplemented with 10 mM Hepes, 2mM glutamine, 0.1mM nonessential amino acids, 10% FBS, 1ng/ml epidermal growth

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factor (EGF) and 2 μ g/ml insulin.

H-358, a lung cancer cell line (kindly provided by J. Minna, NCI-Navy Medical Oncology Branch, Bethesda, MD), and MDA-MB-453 cells, a breast cancer cell line (ATCC Accession No. HTB131), were grown in RPMI containing 10% FBS. MDA-MB-157 (ATCC Accession No. HTB 24) a breast cancer cell line, was grown in IMEM (Gibco BRL) supplemented with 10% FBS and 0.5% Redu-Ser II (Upstate Biotechnology Inc, Lake Placid, NY).

Normal Mammary Epithelial Cells (NMECs) derived from reduction mammoplasties (CC-201 6, Clonetic Corp., San Diego, CA), and 184B5 cells, immortalized mammary epithelial cells (ATCC CRL10317) were cultured in Mammary Epithelial Basal Medium (MEBM, Clonetics, Corp.) supplemented with 1x vitamins, 0.5% FBS, 20 ng/ml EGF, 5 μ g/ml hydrocortisone and 52 μ g/ml bovine pituitary extract (Gudas, et al., Cell Growth Differ., 5:295-304 (1994)).

Immortalized MCF10 cells (kindly provided by S. Brooks, Michigan Cancer Foundation) were cultured in DMEM/F12 (Gibco BRL) supplemented with 2.5% horse serum (Gibco BRL) 10 mM Hepes (Calbiochem, La Jolla, CA), 2 mM glutamine (Biofluids, Rockville, MD), 0.1mM nonessential amino acids, (Gibco BRL) 20 ng/ml EGF (Upstate Biotechnology), 10 μ g/ml insulin (Boehringer Mannheim, Indianapolis, IN), 0.5 μ g/ml hydrocortisone.

293 cells, (ATCC Accession No. CRL 1573), an adenovirus transformed human embryonic kidney cell line was cultured in improved minimal essential medium (Biofluids) supplemented with 2 mM glutamine (Biofluids), 2.5 mcg/ml fungizone (Biofluids), 100U/ml penicillin, 100 mcg/ml streptomycin (100xPen-Strep, Biofluids) and 10% FBS.

AoVSMC (CC-2023, MyoPack-AOSMC, Clonetics Corps., San Diego, CA) were cultured in SmGM, based on the MCDB 131 formulation, and supplemented with 10ng/ml hEGF, 2 mg/ml hFGF, 0.39 ug/ml Dexamethazone, 5% FBS, 50 ug/ml Gentamicin and 50 ng/ml Amphotericin-B.

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EXAMPLE 7**(1) Effect of AdWTp53 on Cell Growth**

The effect of high level wild type p53 expression on the growth of cells having varying p53 status was determined using the following cell lines:

(1) H-358 lung cancer cells, which are devoid of p53 protein (p53 null) (See Takahashi, et al., Science, 246:491-494 (1989));

(2) MDA-MB-231 human breast cancer cells, which express mutant p53 protein (Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)); and

(3) MCF-7 human breast cancer cells which express wild-type p53 protein (Casey, et al., Oncogene, 6:1807-1811 (1991); Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)).

5×10^4 cells of each respective cell line were plated in each well of 6 well tissue culture dishes. After 24 hours, the cells were exposed to 10 pfu/cell of AdWTp53 or AdControl in medium containing 2% fetal bovine serum. After an incubation of 2 hours at 37°C, the serum concentration in the medium was raised to 10% and the incubations continued at 37°C. The cells were trypsinized on each day and counted using a hemacytometer. The cytotoxicity of the adenovirus vectors was assessed using a colorimetric assay as described previously (Skehan, P., J. Natl. Cancer Inst., 82:1107-1112 (1990)). Briefly, 500 cells were plated in each well of 96 well plates. 24 hours later, the cells were exposed to the appropriate cell growth medium. However, in order to facilitate a more efficient infection, the concentration of the serum (if it was a component of the growth medium) was reduced to 2%. Varying doses of adenovirus vectors were included in the incubation medium (several 5-fold dilutions). After a 2 hour incubation at 37°C, the serum concentration was increased to 10% and the cells were incubated for 7 days at 37°C. The cells were fixed by the addition of ice-cold 50% trichloroacetic acid (TCA), which was added onto the top of the medium in each well to a final

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concentration of 10%, the cells were incubated at 4°C for 1 hour, washed five times with water, and allowed to air dry. TCA-fixed cells were stained for 20 minutes with 0.4% (wt/vol) sulforhodamine B (Sigma, St. Louis, MO) dissolved in 1% acetic acid, followed by rinsing four times with 1% acetic acid. An O.D.₅₆₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and was used as a measure of cell number. The percent survival rates of cells exposed to adenovirus vectors were calculated by assuming the survival rate of uninfected cells to be 100%.

As shown in Figures 3A and 3B, infection of H-358 and MDA-MB-231 cells with AdWtp53 completely inhibited cell growth over the four day period examined. In both of these cell lines, the cell number was reduced by day 4 to levels less than half of that present at time 0. In contrast, MCF-7 cells continued to proliferate although at a slower rate than control cells (See Figure 3C). Figures 3A-3C demonstrate that the AdControl virus had very little effect on the growth of these cells.

These results suggested that infection by AdWtp53 had a more profound growth inhibitory effect on cells that were either deficient in p53 or expressed a mutant p53 than on cells that expressed wild-type p53. To confirm these observations, the effects of AdWtp53 on cancer cells that lack p53 expression (H-358, MDA-MB-157) (Takahashi, et al., Science, 246:491-494 (1989); Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)), cancer cells that expressed endogenous mutant p53 (MDA-MB-231, MDA-MB-453) (McGrory, et al., Virology, 163:614-617 (1988); Zakut-Houri, et al., EMBO J., 4:1251-1255 (1985)), cancer cells that expressed wild type p53 (MCF-7), and immortalized and normal cells that expressed wild type p53 (MCF-10, 184B5, NMECs) (J. Gudas, et al. - unpublished data) were investigated. Briefly, each cell line was exposed to increasing concentration of AdWtp53 for 7 days. As shown in Figure 4 and Table 1, cells that are null for expression of p53 were the most sensitive to

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the inhibitory effect of AdWtp53 (IC_{50} for H-358 and MDA157 cells were 0.17 and 0.3 pfu/cell respectively). Cells that express a mutant p53 protein were only slightly less sensitive to the growth inhibitory effects of AdWtp53 (IC_{50} for MDA-MB-231, and MDA-MB-453 were 0.4 and 0.7 pfu/cell respectively). In contrast, immortalized or normal cells that expressed wild type p53 were the most resistant to the cytotoxic effects of AdWtp53, with NMECs being the most resistant (IC_{50} for 184B5, MCF-10, MCF-7 and NMEC were 4.5, 5.5, 30 and 100 pfu/cell respectively) (See Table 1). Thus, cells that express wild type p53 were 5-250 times more resistant to the AdWtp53-mediated inhibitory effect on cell growth when compared with cells expressing no p53 or mutant p53.

(2) β -galactosidase Activity Following Ad.RSV β gal Infection

Because differences in the sensitivity of various cell lines to AdWtp53 could result from either reduced uptake and/or decreased transgene expression, the expression of an adenoviral vector containing the marker gene, β -galactosidase was examined in the following cell lines: NMEC, MCF-7, MCF-10, MDA-MB-453, MDA-MB-231, MDA-MB-157, MDA-MB-453, and H-358.

The cells were plated at a concentration of 2×10^4 cells in each well of a 96 well tissue culture plate. After 24 hours, the cells were exposed to various concentrations of Ad.RSV β gal (0.1-500 pfu/cell) in medium used by each respective cell line, however, the serum concentration (if required) was reduced to 2%. After a 2 hour incubation at 37°C, the serum concentration (if required) was raised to 10%, and the cells were then incubated at 37°C for an additional 24 hours. The cells were washed three times with phosphate buffered saline pH 7.5 (PBS) and lysed in 50 μ l of 0.1M Tris pH 7.5 containing 0.1% Triton X-100. An aliquot (30 μ l) was assayed for β -galactosidase activity using a modified protocol (Stratford-Perricaudet, et al., J. Clin.

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Invest, 90:626-630 (1992)). Samples were transferred to each well of a 96 well plate and treated with 100 μ l of 20 mM Tris pH 7.5 containing 1 mM $MgCl_2$, 450 μ M β -mercaptoethanol, 150 μ M O-nitrophenyl- β -galactopyranoside. Incubations were performed at 37°C for 20 min. and the reaction stopped by the addition of 150 μ l/well of 1 M Na_2CO_3 . The optical density was determined at 420 nm. An O.D.₄₂₀ of 1 was defined as 1 unit of enzyme activity.

Following infection of each cell line at 20 pfu/cell, the enzyme activity in each cell line was in the range of 0.3-0.75 units (See Table 1). Moreover, as shown in Figure 5, each of the cell lines expressed high amounts of p53 when they were exposed to AdWTp53. Therefore, the differences in the sensitivity of killing effects of AdWTp53 cannot be explained by alteration in viral uptake and/or differential expression of the transgene.

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Table 1

Summary of the status of endogenous p53 in various cell lines, IC₅₀ values of AdWtp53 in each of the cell line, β -galactosidase (β -gal) activity in these cells after infecting with Ad.RSV β gal vector, and relative induction of WAF1/Cip1 protein expression following infection with AdWtp53.

Cell line	Endogenous	IC ₅₀	β -gal activity ^c	
Fold induction		p53 status ^a	AdWtp53 ^b	WAF1/Cip1 ^d
H-358	Null	0.17	0.75	105
MDA-MB-157	Null	0.30	0.54	83
MDA-MB-231	Mutant	0.4	0.66	154
MDA-MB-453	Mutant	0.70	0.58	71
MCF-7	Wild type	0.3030	0.30	2.3
184B5	Wild type	4.5	ND	2.4
MCF-10	Wild type	5.5	ND	7.5
NMECs	Wild type	100	0.731	1.2

^a The status of endogenous p53 in each cell line are taken from references 2, 16, 29, 32, 33 and J. Gudas, et al. (unpublished data) as described in the text.

^b These values are estimated from Figure 4.

^c β -galactosidase activity in each cell line was measured after infecting cells with 20 pfu/cell as described.

^d Fold-increase in the WAF1/Cip1 expression represent the AdWtp53-mediated (10 pfu/cell) increase in WAF1/Cip1 protein expression over the uninfected cells.

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EXAMPLE 8**Immunoprecipitation of p53 Protein in Cells Infected with AdWTp53**

Cells (1×10^6) were plated in 10 cm dishes and infected with AdWTp53 or AdControl for 24 hours as described in Example 6. Immunoprecipitations were performed using an anti-p53 antibody essentially as described (Seth, P., et al., Mol. Cell. Biol., 4:1528-1533 (1984)). Briefly, cells were incubated with 3 ml of methionine-free DMEM (Biofluids) containing 5% dialyzed fetal calf serum (Biofluids) and 100 $\mu\text{Ci/ml}$ of [^{35}S]methionine and [^{35}S]cysteine mixture (Express ^{35}S -protein labeling mix, 1000 Ci/mmol, (NEN) for 2 hrs. Cells were washed with ice-cold PBS, and solubilized at 4°C in buffer A (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 0.1% Sodium deoxycholate (Sigma), 0.5% sodium lauryl sulfate (SDS) (Research Genetics, Huntsville, AL), 1 mM phenylmethyl sulfonylfluoride, 10 $\mu\text{g/ml}$ aprotinin, 1.0 $\mu\text{g/ml}$ leupeptin, 1.0 $\mu\text{g/ml}$ pepstatin (all protease inhibitors from Boehringer Mannheim). Aliquots of 500 μl of ^{35}S -labeled lysates were incubated with a 1:50 dilution of anti-p53 monoclonal antibody PAb 1801 (Ab-2) (Oncogene Science, Uniondale, NY) at 4°C for 1 hour, after which 15 μl of protein A/G agarose (Oncogene Science) was added and the incubations were continued for an additional 1 hour with rotation. Samples were then centrifuged at $5000 \times g$ for 5 min, and the pellets were washed successively with buffer A, buffer A containing 1M NaCl, and finally with buffer A again. SDS-gel sample buffer (50 μl) was added, and the samples heated for 5 min at 95°C to elute proteins from the immunoabsorbent. The tubes were centrifuged again at $5000 \times g$ for 5 min to remove protein A/G agarose, and 20 μl aliquots of protein samples were subjected to SDS-polyacrylamide gel electrophoresis. The gels were then dried and exposed to X-ray film as described previously (Seth, et al., Mol. Cell. Biol., 4:1528-1533 (1984)).

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EXAMPLE 9**Western Blot Analysis of p53, WAF1/Cip1 and mdm2 Proteins in Cells Infected with AdWTP53**

In an effort to determine the molecular mechanisms underlying the cytotoxicity of AdWTP53, the expression of two cellular proteins that could play a role in mediating the inhibitory effects of p53 at both the protein and RNA levels was examined (See Example 6). These included WAF1/Cip1, a gene which is induced in cells and inhibits cyclin kinase (Katayose, et al., Am. J. Physiol., 264:L100-L106 (1993); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Fairchild, Cancer Res., 47:5141-5148 (1987)), and mdm2, another p53 inducible gene that can bind p53 and modulate its function (Gudas, et al., Cell Growth Differ., 5:295-304 (1994)).

To demonstrate the effects of AdWTP53 and mdm2 on WAF1/Cip1 protein expression, RNA was prepared from the following cell lines and used in the Western blot analysis:

(1) Null p53 cell lines:

- (a) MDA-MB-157
- (b) H-358

(2) Mutant p53 cell lines:

- (a) MDA-MB-231
- (b) MDA-MB-4533

(3) Wild-type p53:

- (a) MCF-7
- (b) MCF-10
- (c) 184B5
- (d) NMECs

Each respective cell line was plated in 6 cm tissue culture dishes at a concentration of 0.5×10^6 and incubated with AdWTP53 or AdControl for 24 hours as described in Example 2. The cells were then washed three times with ice-cold PBS, scraped and resuspended in 1 ml of 1x SDS-poly acrylamide gel electrophoresis buffer (62 mM Tris pH 6.8, 2 mM ethylenediaminetetraacetate (EDTA), 15%

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sucrose, 10% glycerol, 3% SDS, 0.7 M 2-mercaptoethanol) and
boiled for 10 min. Equal amounts (15 or 50 μ g) of denatured
protein were electrophoresed on SDS-polyacrylamide gels and
transferred to nitrocellulose filters (Gudas, et al., Cell
5 Growth Differ., 5:295-304 (1994)). Filters were blocked
with Tris-buffered saline containing 5% dried milk and 0.1%
Tween 20 (Sigma). Blots were probed with 4 μ g/ml of Ab-2;
and Ab-6 for p53, 4 μ g/ml of EA 10 for WAF1/Cip1, 3 μ g/ml of
IF2 for mdm2, with 3 μ g/ml of Actin (Ab-1) antibody. All
10 antibodies were obtained from Oncogene Science. Following
incubation with the primary antibodies, the blots were
washed with Tris-buffered saline containing 0.1% Tween 20,
incubated with horseradish peroxidase, conjugated to
secondary antibody, and the specific complex was detected by
15 the enhanced chemiluminescence technique according to the
manufacturer's directions (NEN).

As shown in Figure 5, the Western blot analysis
demonstrates that low levels of endogenous p53 were detected
in all cell lines examined except MDA-MB-157 and H-358.
20 However, the level of p53 increased substantially (at least
10-fold) in each cell line following AdWtp53 infection (10
or 50 pfu/cell). In contrast, the amount of p53 increased
little, if at all, above the endogenous p53 protein level in
cells exposed to 50 pfu/cell of AdControl. Because cells
25 exposed to either AdControl or AdWtp53 expressed similar
levels of actin protein, (See Figure 5), increased levels of
p53 following AdWtp53 infection can not be due to loading of
different amounts of proteins or other non-specific
mechanisms.

30 The induction of WAF1/Cip1 expression following
AdWtp53 infection was also examined. As shown in Figure 5,
there was little or no detectable basal level of WAF1/Cip1
in cells that did not express endogenous wild type p53 (MDA-
MB-157, H-358) or in cells that expressed a mutant p53 (MDA-
35 MB-231, MDA-MB-453), basal levels of WAF1/Cip1 were readily
detected in cells that expressed endogenous wild type p53

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(MCF-7, MCF-10, 184B5, NMECs). While exposure of cells to 50 pfu/cell of AdControl did not affect the basal level of WAF1/Cip1 in any of these cells, exposure to 10 or 50 pfu/cell of AdWtp53 resulted in a marked increase in WAF1/Cip1 expression in most of the cell lines (See Figure 5). AdWtp53 infection resulted in greater than 70 fold increase in WAF1/Cip1 protein in cells that lacked endogenous p53 gene expression (MDA-MB-157, H-358) or expressed mutant p53 (MDA-MB-231, MDA-MB-453) (See Table 1). The magnitude of WAF1/Cip1 induction observed following AdWtp53 infection was lower in MCF-7, MCF-10, 184B5 and NMECs; cells that expressed endogenous wild type p53 (See Table 1).

mdm2 protein levels were also determined before and after AdWtp53 infection in each cell line. Basal levels of mdm2 protein were not detectable in cells that were null for p53 or contained mutant p53. Endogenous mdm2 protein bands of approximately 90 kDa and 57 kDa (Gudas, et al., Cell Growth Differ., 5:295-304 (1994); and J. Gudas, et al. - unpublished data) were readily detected in all cells expressing wild type p53, and no difference in the levels of either of mdm2 proteins were observed following infection of cells with AdControl vector. In contrast, following exposure to AdWtp53 at 10 or 50 pfu/cell, there was a marked increase in the levels of high and low molecular weight mdm2 proteins in all cell lines examined - except MCF-7 cells in which AdWtp53-mediated expression of the 57 Kd protein was minimal.

EXAMPLE 10

Northern Blot Analysis of WAF1/Cip1 and mdm2 in Cells Infected with AdWtp53.

To demonstrate the effect of AdWtp53 and mdm2 on WAF1/Cip1 total RNA expression, RNA was prepared from the following cell lines and used in the Northern blot analysis:

- (1) NMEC (normal mammary epithelial cells;

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- (2) MCF-10 (breast cancer cells);
- (3) MCF-7 (breast cancer cells);
- (4) MDA-MB-453 (breast cancer cells);
- (5) MDA-MB-231 (breast cancer cells); and
- (6) MDA-MB-157 (breast cancer cells).

Each respective cell line was plated in 15 cm tissue culture dishes at a concentration of 2×10^6 , and incubated with 10 pfu/cell of adenoviral vectors. After incubation for 24 hours at 37 °C, RNA was extracted by rinsing the cells three times with cold PBS and dissolving them in a 2 ml solution of guanidine isothiocyanate. RNA was purified by centrifugation over a 5.7 M cesium chloride cushion (See Gudas, et al., Cell Growth Differ., 5:295-304 (1994)), fractionated by electrophoresis in agarose gels containing formaldehyde, transferred to Magna NT filters, and cross-linked as described previously in Gudas, et al., Cell Growth Differ., 5:295-304 (1994). Following prehybridization, the filters were hybridized using a 2.1 kb fragment of WAF1/Cip1 or an 800 bp fragment from 36B4. Following hybridization the filters were washed and exposed to X-ray films, and autoradiographs developed as described (See Gudas, et al., Cell Growth Differ., 5:295-304 (1994)).

Since p53 is a DNA binding transcription factor (Skehan, et al., J. Natl. Cancer Inst., 82:1107-1112 (1990)), it was determined whether AdWtp53-mediated induction of WAF1/Cip1 protein was regulated at the level of RNA. The expression of WAF1/Cip1 mRNA was assessed by Northern blot analysis following infection of cells with either AdControl or AdWtp53. As shown in Figure 6, cells devoid of Wtp53 (MDA-MB-157) and cells expressing mutant p53 (MDA-MB-453, MDA-MB-231) had very low levels of WAF1/Cip1 mRNA after infection with AdControl. NMECs, MCF-10 and MCF-7 cells all contained endogenous wild-type p53 and expressed varying levels of WAF1/Cip1 mRNA expression following infection with AdControl. Following infection of AdWtp53, the WAF1/Cip1 mRNA levels in cell lines null for p53 or

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expressing mutant p53 were induced significantly; MDA-MB-453 showed a 7.4 fold increase, MDA-MB-231 showed a 21 fold increase, and MDA-MB-157 showed a 8.2 fold increase. However, when cells expressing wild type p53 were infected with AdWtp53, only MCF-7 cells showed a marked 6 fold increase in WAF1/Cip1 mRNA levels. MCF-10 showed only a 2 fold increase, and NMECs only a 1.2 fold increase in WAF1/Cip1 mRNA following infection with AdWtp53. The level of a control mRNA (36B4) was similar in cells infected with either AdControl or AdWtp53. Thus, the induction of WAF1/Cip1 proteins in cells following infection with AdWtp53 is mediated by an increase in WAF1/Cip1 mRNA.

It was also determined that NMECs, which are the most resistant to killing by AdWtp53, despite the expression of high levels of p53 following AdWtp53 infection, did not undergo apoptosis and showed the smallest increase (1.2 fold) in WAF1/Cip1 induction. Conversely, tumor cells deficient in wild-type p53 or expressing mutant p53 were quite sensitive to the cytotoxic effects of AdWtp53 and showed marked induction of WAF1/Cip1 RNA and protein. Therefore, AdWtp53-mediated cytotoxic effects appeared to be associated with the high expression of WAF1/Cip1. The WAF1/Cip1 gene has been shown to bind to cellular cyclin CDK kinase and thereby inhibit their function (Katayose, et al., Am. J. Physiol., 264: L100-L106 (1993); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Fairchild, et al., Cancer Res., 47:5141-5148 (1987)). This inhibition is manifested in turn by a decrease in the level of phosphorylation of the Rb protein (Seth, et al., Mol. Cell. Biol., 4:1528-1533 (1984)).

EXAMPLE 11

Detection of Nucleosomal DNA Fragmentation in Cells Infected with AdWtp53

To investigate whether the mechanisms of AdWtp53-mediated inhibition of cell growth involved programmed cell

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death (apoptosis), the effect of AdWtp53 on nucleosomal DNA fragmentation was determined after infection of MDA-MB-231 cells (which express endogenous mutant p53), H-358 cells (which are null for p53), and in MCF-7 and NMECs (both of which express endogenous wild type p53).

Each respective cell line was plated at a concentration of 2×10^6 cells in 10 cm tissue culture dishes and incubated with adenoviral vectors (50 pfu/cell) for 24 hours. Both adherent and floating cells were collected together and pelleted by centrifugation at $1800 \times g$ for 5 min (RT-6000B, Du Pont, Boston). Cell pellets were rinsed with cold PBS and low molecular weight DNA was prepared by a modified Hirt extraction method as described in Rosenfeld, et al., Hum. Gene Ther., 5:331-342 (1994). Briefly, pellets were lysed in 1 ml of 10 mM Tris, 10 mM EDTA disodium pH 7.4 (Research Genetics), 0.6% SDS (Research Genetics) and 0.2 mg/ml proteinase K (Boehringer Mannheim). Samples were incubated at 55°C for 5 hours, low molecular weight DNA was prepared by the Hirt extraction method as described in Rosenfeld, et al., Hum. Gene Ther., 5:331-342 (1994) and the evaluated by electrophoresis on a 2% agarose gel.

As shown in Figure 7, 24 hours following exposure of MDA-MB-231 cells to 50 pfu/cell of AdWtp53, several lower molecular weight DNA bands (DNA laddering of approximately 145 bp) in the range of 145-1050 bp were observed. This observation is characteristic of cells undergoing apoptosis. In contrast, exposure of MDA-MB-231 cells to AdControl or mock infection of these cells produced no detectable DNA laddering (See Figure 7). When MCF-7 cells or NMECs were exposed to AdWtp53 at 50 pfu/cell, no DNA laddering was observed (See Figure 7). Infection of MCF-7 cells with 1000 pfu/cell also did not demonstrate DNA laddering.

These results indicate that tumor cells null for p53 or expressing an endogenous mutant p53 undergo apoptosis following exposure to AdWtp53, while tumor cells or normal cells expressing wild type p53 are resistant to apoptosis.

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EXAMPLE 12**AdWtp53-mediated Synthesis of p53 protein**

To determine if AdWtp53 expresses p53 protein in tumor cells, the lung tumor cell line H-358, which lacks endogenous p53 (Takahashi, et al., Science, 246, 491-494 (1989)) was exposed to various concentrations of either AdControl or AdWtp53 for 24 hours. Following infection, immunoprecipitation of p53 was performed as described in Example 4. As shown in Figure 2A, there was no detectable p53 in H358 cells infected with AdControl. In contrast, p53 protein was easily detected by immunoprecipitation in cells infected with 1 pfu/cell of AdWtp53. Furthermore, the amount of immunoprecipitable p53 protein increased with increasing concentrations of AdWtp53 vector.

To investigate adenoviral-mediated p53 expression in breast tumor cells, several different mammary cell lines (MCF-7, MCF-10, Adr^R MCF-7, MDA-MB-231) were exposed to AdWtp53, and the synthesis of p53 protein assessed by immunoprecipitation. As shown in Figure 2B, MCF-10, MCF-7, Adr^R MCF-7 and MDA-MB-231 cells expressed low levels of endogenous p53. However, following exposure of the cells to 10 pfu/cell of AdWtp53, a marked increase in the rate of p53 protein synthesis was observed in the infected cells. In contrast, infection with AdControl did not result in any increase in p53 expression above that present in uninfected cells. These results were also confirmed by Western blot analysis (see Example 5) and indicate that AdWtp53 can infect both human mammary and lung cells. Moreover, infection with AdWtp53 resulted in high levels of p53 expression in these cells.

EXAMPLE 13**WAF1/Cip1 Expression/Induction Following AdWtp53 and AdWAF1 Infection.**

AdWtp53-mediated p53 expression and its consequence on WAF1/Cip1 induction were examined in cells

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infected with AdWTP53. In parallel experiments, AdWAF1-mediated WAF1/Cip1 expression was also examined in cells infected with AdWAF1. The cell lines studied included: (1) H-358 lung cancer cells, which are devoid of p53 expression (p53 null) (Takahashi, et al., Science 246:491-494 (1989)); MDA-MB-231 human breast cancer cells which express mutant p53 (Casey, et al., Oncogene 6:1807-1811 (1991)); MCF-7 human breast cancer cells which express wild type p53 (Casey, et al., Oncogene 6:1807-1811 (1991)) and normal mammary epithelial cells (NMECs) which also express endogenous wild type p53 (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). The cells were cultured and maintained as set forth in Example 2.

EXAMPLE 14

Western Blot Analysis.

Cells (1×10^6) were plated in 10 cm tissue culture dishes and incubated with adenoviral vectors for 48 hours as described in Example 2. The cells were then scraped and cell lysates subjected to Western blot analysis as previously described (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). The blots were probed with 3 μ g/ml of Ab-2 and Ab-6 for p53, 3 μ g/ml of EA 10 for WAF1/Cip1, with 3 μ g/ml of Actin (Ab-1) antibody. All antibodies were obtained from Oncogene Science (Uniondale, NY). The blots were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with horse radish peroxidase conjugated to secondary antibody and specific complex detected by the enhanced chemiluminescence technique according to the manufacturer's directions (Amersham, Arlington Heights, IL).

The Western blot analysis demonstrates that the level of p53 increased substantially (at least 10-fold) following AdWTP53 (50 pfu/cell) infection in each cell line (Figure 2, Top Panel); and p53 expression in turn induced high levels of WAF1/Cip1 protein in all the cell lines.

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(Figure 9, Middle Panel). Following infection with AdWAF1 (50 pfu/cell), a high level expression of WAF1/Cip1 protein was also observed. (Figure 9, Middle Panel). More importantly, WAF1/Cip1 expression following AdWAF1 infection was comparable to the levels induced by AdWtp53 (50 pfu/cell) infection. In these experiments, AdControl vector did not increase p53 or WAF1/Cip1 expression; and no change in actin protein level was detected in any of the cells lines following infection with either AdControl, AdWtp53 or AdWAF1 (Figure 9, Lower Panel).

EXAMPLE 15

Cell Growth and Cytotoxicity Assays and Effects of AdWtp53 and AdWAF1 on Cell Growth/Killing.

For cell growth measurements, 5×10^4 cells were plated in each well of 6 well tissue culture dishes. After 24 hours, cells were exposed to adenoviral vectors (10 pfu/cell) and incubations continued at 37°C. The cells were trypsinized on each day and counted using a hemacytometer. Cytotoxicity of adenovirus vectors was also assessed after plating cells in 96 well plates (500 cells/well). The cells were incubated with various doses (0- 10^4 pfu/cell) of adenovirus vectors for 7 days at 37°C. The cells were fixed with trichloroacetic acid and stained with 0.4% (wt/vol) sulforhodamine B (Sigma, St. Louis, MO) essentially as described previously (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). An O.D.₅₆₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and used as a measure of cell number.

The cytotoxic effects of p53 and WAF1/Cip1 overexpression were investigated in cells infected with AdWtp53 and AdWAF1. Infection of H-358 and MDA-MB-231 cells with AdWtp53 completely inhibited cell growth over the seven day period examined (Figures 10A and 10B), and the cell number was reduced by day 7 to levels less than half of that present at time 0. Following AdWtp53 infection, MCF-7 cells

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continued to proliferate, although at a slower rate than the control cells (Figure 10C). The growth inhibitory effect of AdWAF1 was much weaker than AdWtp53 in H-358 and MDA-MB-231 cells, but was comparable to that of AdWtp53 in MCF-7 cells (Figures 10A-10C). In cytotoxicity assays, AdWtp53 was about 30-100 times more toxic than AdWAF1 in H-358 and MDA-MB-231 cells, and nearly equally toxic in MCF-7 and NMECs. Thus, the AdWtp53 infection had a more profound growth inhibitory effect as compared to AdWAF1 on cells that were either deficient in p53 or expressed a mutant endogenous p53. However, the growth inhibitory effects of AdWtp53 and AdWAF1 on cells expressing wild-type endogenous p53 were similar, though much weaker.

EXAMPLE 16

Cell Cycle Analysis; Nucleosomal DNA Fragmentation Analysis and the Effect of AdWtp53 and AdWAF1 on Cell Cycle and Apoptosis.

Cells were plated in 6 well tissue culture dishes (2×10^5 cells/well) and infected with adenoviral vectors (50 pfu/cell) for 48 hours. The cells were harvested by trypsinization and resuspended at a concentration of 2×10^5 cells/ml in medium containing 10% FBS, and stored frozen until analyzed. Samples were stained for DNA cell cycle analysis using the rapid nuclear isolation procedure (Wersto, R. P., and Stetler-Stevenson, M. A., Cytometry (in press, 1995)). DNA content was measured using a FACScan flow cytometer (Becton-Dickinson, Mountain view, CA). Cell cycle analysis of the resulting DNA histograms of cell number versus integrated red fluorescence was performed with Multicycle (Phoneix Flow Systems, Dan Diego, CA) using a zero order polynomial to model the S-phase fraction. Debris, cell aggregates, and Go/1 doublets were removed from the cell cycle analysis by software algorithms (Wersto, R. P., and Stetler-Stevenson, M. A., Cytometry (in press, 1995)).

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Cells (2×10^6) were plated in 15 cm dishes and incubated with adenovirus vectors (50 pfu/cell) for 48 hours. Both adherent and floating cells were collected and pelleted by centrifugation at $1800 \times g$ for 5 min (RT-6000B, Du Pont, Boston). Low molecular weight DNA was prepared by a modified Hirt extraction method described previously (Katayose, et al., Clin. Cancer Res. (Submitted 1995); Rosenfeld, et al., Hum. Gene Ther. 5:331-342 (1994)) and evaluated on 2.5% agarose gel electrophoresis.

To investigate the mechanisms of the differential cytotoxicities mediated by wild-type p53 and WAF1/Cip1 overexpression, the effects of AdWtp53 and AdWAF1 infection on the two major growth regulatory mechanisms, cell cycle and apoptosis, were examined.

DNA cell cycle histograms were used to evaluate the percentage distribution of cells in various cell cycle stages in uninfected, AdControl infected, AdWtp53 infected, and AdWAF1 infected cells, and are shown in Figures 11 and 12. In MDA-MB-231 cells (Figures 11A-D; Figure 12A) no effect on cell cycle distribution was observed following infection with AdControl. However, following infection with AdWtp53, a slight increase in cells in G1 phase (from 55 % to 58 %) but a significant decrease in S phase cells (from 24% to 5 %), and a significant increase in the percentage of G2 + M cells from 21 % to 37 % were observed (Figure 11C; Figure 12A). On the other hand, AdWAF1 infection led to an increase in the cell percentage in G1 phase from 55% to 91%, reduction in percentage of cells in S phase from 24% to 2%, and a decline in G2 + M phase from 21% to 7% (Figure 11D; Figure 12A). Thus, while both AdWtp53 and AdWAF1 infection led to a decline in the S phase cells, AdWtp53 caused an accumulation of cells in G2/M phase and AdWAF1 infection resulted in the cell accumulation in G1 phase. These results indicate that WAF1/Cip1 overexpression arrests the cell cycle at G1/S boundary, while p53-overexpression can cause a cell cycle arrest at G2/M stage.

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AdWtp53 and AdWAF1 produced similar effects in H-358 cells. AdWtp53 produced a reduction in S phase and increase in G2 + M phase; while AdWAF1 produced a reduction in S phase and increase in G1 phase (Figure 12B). In MCF-7 and NMECs, AdWtp53 and AdWAF1 infection resulted in declines in the percentage of cells in S phase in MCF-7 and NMECs. However, no significant change in proportion of cells in G2 + M or G1 was apparent following AdWtp53 or AdWAF1 infection of these cells (Figures 12C and 12D).

The effects of AdWtp53 and AdWAF1 on apoptosis was also examined by assaying the nucleosomal DNA fragmentation and cell cycle analyses of both adherent and floating cells. As shown in Figure 13, 24 hours following exposure of H-358 and MDA-MB-231 cells to 50 pfu/cell of AdWtp53, several lower molecular weight DNA bands (DNA laddering of approximately 145 bp) in the range of 145-1050 bp were observed. These DNA fragments are characteristic of cells undergoing apoptosis (Figure 13B). In contrast, exposure of MDA-MB-231 and H-358 cells to AdWAF1, AdControl or mock infection of these cells produced no detectable DNA laddering (Figures 13A and 13B). Furthermore, following exposure of MCF-7 cells to AdWtp53 or AdWAF1 at 50 pfu/cell, no DNA laddering was observed (Figure 13C). NMECs also did not undergo apoptosis following AdWtp53 or AdWAF1 infection.

Cell cycle analysis of both adherent and floating cells showed the appearance of apoptotic cells following AdWtp53 infection in H-358 and MDA-MB-231 as determined by DNA fragmentation on FACS analysis. However, AdWAF1-infection failed to induce apoptotic cells following infection of H-358 and MDA-MB-231 cells (cell cycle pattern of MDA-MB-231 following infection with AdWtp53 and AdWAF1 is shown in Figures 13D and 13E respectively; the apoptotic cells peak is indicated by an arrow). Cell cycle profiles of MCF-7 and NMECs also failed to detect the presence of dead cells following infection with either of the adenoviral vectors.

These results indicate that tumor cells null for p53

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(H-358) or expressing an endogenous mutant p53 (MDA-MB-231) undergo apoptosis following exposure to AdWtp53; while tumor cells (MCF-7) or normal cells (NMECs) expressing wild-type p53 appeared to be resistant to AdWtp53 induced apoptosis. Moreover, overexpression of WAF1 protein following AdWAF1 infection does not appear to mediate apoptosis even in cells null for p53 or expressing endogenous mutant p53. These results suggest that while WAF1/Cip1 protein overexpression can cause cell cycle arrest, overexpression of this protein is not sufficient to induce apoptosis.

EXAMPLE 17

Cell Cycle Analysis; Nucleosomal DNA Fragmentation Analysis and the Effect of Adp27 and Adp16 on Cell Cycle and Apoptosis.

A. Cell Cycle Analysis.

Cells were plated in 6-well dishes (2×10^5 cells/well) and infected with adenoviral vectors (50pfu/cell) for 48 hours. Cells were harvested by trypsinization and resuspended at a concentration of 2×10^5 cells/ml in 100% FBS and stored frozen until analyzed. Samples were stained for DNA cell cycle analysis using the previously described procedure. DNA content was measured using a FACSscan flow cytometer (Becton-Dickenson, Mountain View, CA.). Cell cycle analysis of the resulting DNA histograms of cell number versus integrated red fluorescence was performed with Multicycle (Phoenix Flow Systems, San Diego, CA.) using a zero order polynomial to model the S-phase fraction. Debris, cell aggregates, and G0/G1 doublets were removed from the cell cycle analysis by software algorithms.

B. Nucleosomal DNA Fragmentation (Apoptosis) Analysis.

Cells (2×10^6) were plated in 15 cm dishes and the next day incubated with adenovirus vectors (50 pfu/cell) for 24 hours. Both adherent and floating cells were collected and pelleted by centrifugation at $1800 \times g$ for 5 minutes (RT-6000B, Du Pont, Boston). Low molecular weight DNA was

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prepared by a modified Hirt extraction method described previously, and evaluated on 2.5% agarose gel electrophoresis.

C. Cyclin Kinase Assays.

4 x 10⁶ cells were plated in 15 cm dishes. The next day the cells were infected with recombinant adenoviruses (50 pfu/cell) for 24 hours. The cells were then harvested and lysed in a buffer. For cdk2 kinase activity, lysates were immunoprecipitated by anti-cdc2. For cdc2 kinase, cell lysates were immunoprecipitated. For cdc2-cyclin B-1 dependent kinase, lysates were precipitated with anti-cyclin B1. In brief, cell lysates were incubated with 1 ug primary antibody for 1 hour at 40C. Immune complexes were collected on protein A-Sepharose beads. The beads were washed three times with EBC buffer, three times with kinase reaction buffer (20 mM Tris-HCL pH 7.5, 4 mM MgCl₂). The beads were then resuspended in kinase assay mixture containing 80 uM (³²P-ATP), histone H1 (2 ug) (Gibco-BRL). After incubation at 37°C, the reaction was stopped by the addition of 2X Laemli SDS sample buffer. Proteins were separated on 10% SDS-polyacrylamide gels, and the gels were then dried and autoradiographed.

EXAMPLE 18

Ad.RSV8gal Expression in Breast Tumor and Bone Marrow Cells.

A. Cells and Cell Culture.

Breast cancer cell line MDA-MB-231 (ATCC HTB 26) was cultured in Minimal Essential Medium (Zn++ option) containing 10% Fetal Bovine Serum (FBS) (Gibco BRL, Gaithersburg, MD). 293 cells (ATCC CRL 1573), an adenoviral transformed human kidney cell line, was propagated in improved minimal essential medium (Biofluids, Rockville, MD) containing 10% FBS. Human bone marrow cells were obtained from normal donors. Approximately 10 ml of bone marrow aspirates were collected in a 20 ml syringe containing preservative free heparin (Lymphomed, Deerfield, IL) and

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then diluted in Hanks' Balanced Salt Solution without CaCl_2 or MgCl_2 (HBBS) (Gibco-BRL, Gaithersburg, MD). Cells with a density of $\leq 1.077 \text{ g/cm}^3$ were separated on a Ficoll-sodium diatrizoate gradient (LSM, Organon Teknika Corp., Durham, NC), washed three times with HBBS, and suspended in an enriched Iscove's Modified Dulbeccos' Medium (Gibco BRL, Gaithersburg, MD) (Clarke, et al., Nature 362:849-852 (1993)).

B. Adenoviral Vectors

dl312, a replication-deficient mutant of adenovirus, (Lowe, et al., Nature 362:847-849 (1993)) Ad.RSV β gal vector (a recombinant Ad vector expressing β -gal gene (Dulic, et al., Cell 76:1013-1023 (1994))), and AdWtp53, a replication-deficient recombinant adenoviral vector expressing human wild-type p53 were propagated in 293 cells and purified by two rounds of CsCl_2 density centrifugation. Adenovirus titers were determined from viral stocks and stored in Tris-Cl pH 7.5 buffer containing 20% glycerol.

C. Plasmid DNAs

A plasmid expressing the bacterial β -galactosidase gene driven by cytomegalovirus early gene promoter, (CMV β -gal) (Nelson, W. G. and Kastan, M. B., Mol. Cell. Biol. 14:1815-1823 (1994)) was used to measure the transfection efficiency of cells. Adenoviral-mediated enhancement of toxicity of plasmid DNA was determined using plasmid pULI100, containing the Pseudomonas exotoxin catalytic domain (minus secretory signals) driven by a CMV promoter (Michalovitz, D., Halevy, O. and Oren, M., Cell, 62:671-680 (1990)).

D. Ad Infections

Human breast cancer cells were plated at the appropriate density. After 24 hours, the medium was changed to OPTI-MEM (Gibco-BRL, Gaithersburg, MD) and the cells were infected with various multiplicity of infection (moi) of adenovirus. After 2 hours, FBS was added to a final concentration of 10% and the incubation continued at 37°C. For human bone marrow cells and human CD34^+ cells, freshly

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isolated cells were plated in OPTI-MEM and infected with increasing moi of adenoviral vectors. After 2 hours, FBS was added to the final concentration of 10% and the incubation continued at 37°C. Other experimental details such as the number of cells, moi of adenovirus, and the length of incubations are described below for each experiment.

E. Estimation of Adenovirus Receptor Number

Receptor numbers for adenovirus were determined by scatchard analysis of ^{35}S -dI312 binding to cells as described previously. (See Seth, P., Rosenfeld, M., Higginbotham, J. and Crystal, R. G., J. Virol. 68:933-940 (1994)). In brief, 0.2×10^6 cells were used to bind ^{35}S -dI312 adenovirus (10^4 cpm, $0.1 \mu\text{g}$ adenovirus protein) at 40C for 1 hour in the presence of unlabeled dI312 (0 - $100 \mu\text{g}$). Scatchard plots were drawn as described previously. (See Seth, P., Rosenfeld, M., Higginbotham, J. and Crystal, R. G., J. Virol. 68:933-940 (1994)). Binding assays were conducted in triplicate and the mean taken. Table 2 sets forth the receptor numbers for adenoviruses on breast cancer cells and human bone marrow cells.

D. Ad.RSV β gal Expression.

Breast cancer cells and human bone marrow cells (2×10^4) were plated in 96 well plates and infected with increasing concentrations of Ad.RSV β gal (0.06 - 5000 pfu/cell) for 24 hours. Cells were then lysed in $100 \mu\text{l}$ of 20 mM Tris-HCl, containing 0.1% Triton X 100 and $30 \mu\text{l}$ aliquots used to determine β -gal activity by a calorimetric assay as described previously. An O.D.₄₂₀ of 1 was defined as 1 unit of β -gal activity.

For detecting β -gal expression in individual cells, cells (2×10^4) were plated in 60 mm dish, and infected with 100 pfu/cell Ad.RSV β -gal. Following a 24 hour infection, breast cancer cells were then fixed in phosphate buffered saline, pH 7.5 (PBS) containing 0.5% formaldehyde (mallinckrodt, Inc, Paris, KY) and 0.2% glutaraldehyde

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(Polysciences, Inc, Warrington, PA) at 4°C for 5 min. After washing with PBS, cells were then incubated in a solution containing 0.5 mg/ml X-gal, 10 mM K₄FeCN₆, 10 mM K₃FeCN₆, 10 mM MgCl₂ for 30 min at 37°C to develop the blue color. For CD34⁺ cells the same protocol was used except cells were centrifuged at 3,000 rpm in an eppendorf centrifuge between washing, fixing and staining steps. Cells were photographed using 2000 x magnification.

TABLE 2

**NUMBER OF ADENOVIRUS RECEPTORS ON
BREAST CANCER CELLS AND HUMAN BONE MARROW CELLS**

<u>CELL LINE</u>	<u>RECEPTOR NUMBER</u>	
	<u>HIGH AFFINITY</u>	<u>LOW AFFINITY</u>
(1) MDA-MB-231	6.2 x 10 ³	1.1 x 10 ³
(2) MCF-7	5.3 x 10 ³	7 x 10 ²
(3) MDA-MB-453	7.5 x 10 ³	1.8 x 10 ³
(4) Human Bone Marrow	Undetectable	Undetectable

Cells were exposed to ³⁵S-dI312 adenovirus and the adenovirus receptor number was estimated from the scatchard plots drawn from the binding experiments as described in Example 14.

EXAMPLE 19

AdWtp53-mediated Cytotoxicity Assays and Cytotoxic Effects of AdWtp53 in Breast Tumor and Bone Marrow Cells.

Freshly trypsinized breast cancer cells were mixed with CD34⁺ human bone marrow cells and exposed to different moi of AdWtp53 for 2 hours. Cell cultures were then split; half of the cells (500 cells) were plated in 60 mm dishes and grown in IMEM containing 10 % FBS to form colonies of breast cancer cells. After 14 days the colonies were stained with 5 mM methylene blue, and counted. The other half (500 cells) of the cell suspension was cultured in 0.8 % methyl cellulose medium containing 5 % PHA-LCM (Stem Cell

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Technologies, Vancouver, BC) to determine colony forming units of granulocytes/macrophages. One ml of methyl cellulose medium containing cells was placed in a 35 mm gridded tissue culture dish (Nunc, Naperville, IL), incubated at 37°C, and the CFU-GM colonies counted after 14 days.

Further, the cytotoxic effects of an Ad vector expressing human wild type p53 (AdWtp53) were determined. AdWtp53 was shown to be cytotoxic to breast cancer cells. Following infection of MDA-MB-231 breast cancer cells and CD 34⁺ bone marrow cells with increasing moi of AdWtp53, the cytotoxicity of AdWtp53 was measured by colony forming assays. As shown in Figure 16, following infection of MDA-MB-231 cells with AdWtp53, significant cytotoxicity (about 55% decline in colony numbers) was observed at an moi of 8 pfu/cell. At moi 100 pfu/cell or higher, essentially no colony formation was observed. Bone marrow cells, on the other hand, were much more resistant to killing by AdWtp53. At an moi of up to 1000 pfu/cell, there was essentially no decline in CFU-GM. However, at a very high moi ($\geq 10,000$ pfu/cell) of AdWtp53 there was about 50% reduction in colony numbers (Figure 16). These results indicate that MDA-MB-231 breast cancer cells are at least 3 orders of magnitude more sensitive to the cytotoxic effects of AdWtp53 compared to human CD34⁺ human bone marrow cells.

EXAMPLE 20

Ad and Lipofectamine-mediated Enhancement of CMV β -gal Expression and Ad-mediated Transfection of a CMV β -gal Plasmid in Breast Tumor and Bone Marrow Cells.

Cells (2×10^4) were plated in 96 well plates and exposed to CMV β -gal plasmid (1 μ g) in the absence and presence of dl312 (0.01-100 PFU/cell). To test the effects of lipofectamine, CMV β -gal plasmid DNA (1 μ g) was pre-incubated with different concentrations of lipofectamine (1 μ g) at room temperature for 20 min and used for transfection assays. Following exposure of cells to these reagents for

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2 hours, FBS was added for a final concentration of 10% and the cells incubated at 37°C for 24 hours. Breast tumor cells were then washed twice with PBS and lysed in 20 mM Tris-Cl pH 7.5 containing 0.1% Triton X-100. The same protocol was used for bone marrow cells except the cells were centrifuged at 3,000 rpm, washed twice with PBS and then lysed in the lysis buffer. Aliquots (50 μ l) of cell lysates were used to determine β -gal activity.

Using CMV β -gal, a plasmid expressing the β -gal gene, the adenoviral-mediated enhancement of the plasmid DNA delivery to MDA-MB-231 and bone marrow cells was determined. As shown in Figure 17, MDA-MB-231 cells transfected with plasmid DNA alone, expressed low levels (< 0.01 unit) of β -gal activity. However, in the presence of an increasing concentration of dl312, there was an increase in β -galactosidase activity. The enhancement of the β -gal plasmid expression in this breast cancer cell line was dependent upon the concentration of dl312 used, with maximum β -galactosidase expression (0.65 unit) observed in the presence of 100 pfu/cell. β -galactosidase activity was undetectable in bone marrow cells in the presence of CMV β -gal plasmid alone or in the presence of CMV β -gal plasmid and low concentrations of dl312. Only at a moi of 100 pfu/cell of dl312 was there any detectable β -gal activity (0.007 unit) observed in bone marrow cells (Figure 17). Thus, at an moi of 100 pfu/cell, dl312 infection enhanced the expression of β -gal plasmid DNA in MDA-MB-231 cells 100-fold more than the expression in bone marrow cells.

It is known that adenoviral-mediated increase in plasmid DNA uptake and expression are augmented by the addition of cationic molecules. Therefore, the effect of the polycationic liposome lipofectamine on dl312-mediated CMV β -gal plasmid expression in both human breast cancer cells and human bone marrow cells was determined. As shown in Table 3, dl312 or lipofectamine, when used individually, increased the β -gal activity in MDA-MB-231 cells (0.65 and

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0.58 unit respectively). Furthermore, the presence of both dl312 and lipofectamine increased the β -galactosidase expression in MDA-MB-231 cells 3.8 units (Table 3). Conversely, bone marrow cells showed very little β -galactosidase activity (0.007 and 0.023 units respectively) in the presence of either dl312 (100 pfu/cell) or lipofectamine alone. Even in the presence of both dl312 and lipofectamine, only 0.04 unit of β -gal activity was obtained (Table 3). This level of activity is approximately 100-fold less than that obtained in MDA-MB-231 cells.

Table 3

dl312 and Lipofectamine-Mediated Transfection of CMV β -gal Plasmid in MDA-MB-231 and Human Bone Marrow Cells.

Treatment	β -gal activity (MDA-MB-231 cells)	β -gal activity (Bone marrow cells)
DNA alone	0.001	0.006
DNA + dl312	0.650	0.007
DNA + lipofectamine	0.580	0.023
DNA + dl312 + lipofectamine	3.800	0.040

Cells (2×10^5) were transfected with CMV β -gal plasmid (5 μ g/ml) in the absence and presence of dl312 (10 pfu/cell) and lipofectamine (1 μ g/ μ g DNA) for 24 h. β -gal activity was then estimated as described in Example 14. The values shown are the average of the triplicate determinations.

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EXAMPLE 21

Adenovirus and Lipofectamine-mediated Enhancement of pULI100 Cytotoxicity and Adenoviral-mediated Enhancement of the Cytotoxicity of a Plasmid DNA Expressing Pseudomonas Exotoxin Gene in Breast Tumor and Bone Marrow Cells.

Breast tumor cells were plated in 96 well plates (500 cells/well) and exposed to pULI100 plasmid (1 μ g/well), in the absence or presence of different concentrations of dl312 (0.32-1000 pfu/cell) for 24 hours. To examine the effects of lipofectamine, plasmid DNA was pre-incubated with lipofectamine (1 μ g lipofectamine/1 μ g plasmid DNA). The cells were then exposed to these reagents in OPTI-MEM for 2 hours, after which the serum concentration was raised to 10%. The cells were incubated for 7 days at 37°C. The cells were then fixed in 10% TCA, stained with 0.4% sulforhodamine B (Sigma, St. Louis, MO) and as a measure of cell number, the O.D.₅₆₄ was taken using Bio Kinetic Reader EL 340 (Bio-Tek Instruments). The survival rates of each experimental condition were calculated assuming 100% survival of untreated control cells. The survival rate of CD34+ cells following transfection in the presence of dl312 and pULI100 was estimated by the clonogenic assays described above.

Further, it was determined whether low doses of dl312 could enhance the delivery of a plasmid DNA coding for a toxin gene as measured by the cytotoxicity. To test this, MDA-MB-231 breast tumor cells were transfected with pULI100 DNA, a plasmid containing the cDNA encoding for the catalytic component of Pseudomonas exotoxin which is cytotoxic to cells. Transfections were conducted in the absence and presence of dl312 and/or lipofectamine, and cell survival was examined. As shown in Figure 17, exposure of MDA-MB-231 cells to pULI100 (1 μ g) or a fixed concentration of either dl312 (10 pfu/cell) alone or lipofectamine (1 μ g) alone did not result in any significant cell killing. However, in the presence of either dl312 or lipofectamine,

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pULI100 transfection resulted in > 70 % killing of MDA-MB-231 cells. When pULI100 transfections were performed in the presence of both dl312 and lipofectamine, greater than 95% cell killing was observed (See Figure 18). On the other hand, when human bone marrow cells were exposed to similar concentrations of pULI100 plasmid alone or in combination with dl312 and lipofectamine, less than 5% cells appeared to be killed (See Figure 18). Even when a combination of 100 pfu/cell of AdWtp53, pULI100 plasmid and lipofectamine was used, while CD34+ bone marrow cells were fairly resistant to killing (in colonogenic assays only a decline of about 20 % colony number was obtained), in MDA-MB-231 cells, AdWtp53 alone caused 100% reduction in the colony number. These results indicate that while exposure of breast tumor cells to a low concentration of adenovirus, lipofectamine, and a plasmid coding for a toxin gene are very cytotoxic to breast tumor cells, human bone marrow cells are relatively resistant to these treatments.

EXAMPLE 22

AdCD-Mediated Cytosine Deaminase Assays and Cytotoxic Effects of AdCD in Breast Tumor Cells.

A. Cytotoxicity Assays.

Cytotoxicity of adenovirus vectors was assayed after plating cells in 96 well plates (500 cells/well). Cells were incubated with various doses ($0-10^4$ pfu/cell) of adenovirus vectors in the absence and presence of different concentrations of 5-Fluoro-cytosine (5-FC) for 7 days at 37°C. The cells were fixed with tricholoacetic acid and stained with 0.4% (wt/vol.) sulforhodamine B (Sigma, St. Louis, Mo.) essentially as described. An O.D.₅₆₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and used as a measure of cell number. In the absence of 5-FC, there was no detectable basal activity of CD in either mock infected or AdControl infected cells (Table 4). However, following infection of cancer cells

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with AdCD, very high CD activities were observed. CD expression was dependent upon the dose of AdCD used (See Table 4). In the presence of different concentrations of 5-FC, up to 100 pfu/cells AdCD had no cytotoxicity in either MDA-MB-231 or MCF-7 cells. Similarly, when cells were exposed to different concentrations of 5-FC alone, no significant toxicity was observed. However, when the cytotoxicity assays were performed in the presence of increasing concentrations of AdCD and 5-FC, significant increases (up to 2-3 log) increases in the cytotoxicities of AdCD and 5-FC were detected. These results indicated that the combination of AdCD and 5-FC was cytotoxic to breast cancer cells. However, when cytotoxicity assays were conducted in the presence of AdControl, no significant enhancement of 5-FC cytotoxicity was observed indicating the specificity of AdCD.

Table 4
AdCD-Mediated CD Activity in Breast Cancer Cells

Cell line	CD activity				
	UI	AdControl (10 pfu/cell)	AdCD (10 pfu/cell)	AdControl (100 pfu/cell)	AdCD (100 pfu/cell)
MDA-MB-231	0	0	53170	0	584347
MCF-7	0	0	83246	0	1117224

B. Bystander Effects of AdDC.

To determine if bystander effects contributed to the overall toxicity of AdCD, MDA-MB-231 cells were infected with AdCD (10 pfu/cell) and mixed in different ratios with mock infected cells. Briefly, cells were plated in 10 cm dishes (1×10^6 cells/dish). 24 hours later the cells were infected with either AdControl (10 pfu/cell) or AdCD (10

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pfu/cell). The next day, AdCD-infected cells were mixed with uninfected cells in varying ratios (0.0001 to 100%) and plated in 96-well dishes. A total 20,000 cells were plated in each well. Immediately after plating the cells, 5-FC was added (1-5 mM) and the cells were incubated at 37°C for 5 days. The cell viability was estimated using a MTT assay as described previously. In the presence of 5 mM 5-FC alone, the uninfected cells were 100% viable. However, when the cells were incubated with 5-FC, and either 100, 50, 20 or 10% of the infected cells; the viability of the total cell population was reduced to 0%. However, when the proportion of infected cells was less than 10%, some viable cells were still present at the end of the 5-day period. From these results it appears that to kill 100% of the population, only about 10% of the cells need to be infected by AdCD, which is likely to be due to bystander effects of the AdCD in the presence of 5-FC.

C. Biochemical Measurement of 5-FC Conversion to 5-FU.

Breast cancer cells were plated in 10 cm dishes (2×10^6 cells/dish). The next day the cells were infected with either AdCD or AdControl for 24 hours. Cells were then washed with PBS, scraped and suspended in buffer. Cytosine deaminase activity was then measured.

EXAMPLE 23

Effect of AdCD Infection in Human Xenografts in Nude Mice.

In vivo effects of AdCD were examined in human breast tumors MDA-MB-231 grown as xenografts in nude mice. MDA-MB-231 cells (5×10^6) were injected in nude mice subcutaneously. After 2 weeks, palpable tumors were visible. A single injection of AdCD or AdControl (10^9 pfu in 0.1 ml) was given intratumorally. Immediately after this, the animals were administered with 5-FC (5 mg/kg body weight). 5-FC was administered twice each day for 5 days. Tumor sizes were measured each week. As shown in Figure 20, the tumors of animals which received AdControl or AdCD alone

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continued to grow until day 21. However, further development of tumors of animals which received AdCD in conjunction with 5-FC was significantly inhibited. In contrast, there was no significant effect on the further growth of the tumors of animals which received AdCD and 5-FC.

EXAMPLE 24

Effect of AdWtp53 Infection in Human Xenografts in Nude Mice.

A. Apoptosis Assays.

The effects of p53 overexpression on apoptosis were investigated in MDA-MB-231 human breast cancer cells using terminal deoxyribonucleotidyl transferase immunostaining. MDA-MB-231 cells were plated in 10 cm dishes (2×10^6 /dish) and 24 hour later, medium was changed to IMEM containing 2% FBS. Cells were infected with either AdControl or AdWtp53 (50 pfu/cell). After 2 hours, the serum concentration was raised to 10% and incubation continued at 37°C for another 24 hours. Cells were harvested, fixed in 70% ethanol, and apoptotic cells detected via a terminal transferase reaction using Apotag kit (Oncor, Gaithersburg, Maryland). Cells were photographed using a fluorescent microscope (1000x magnification). As shown in Figure 19, following infection with AdControl, only a very few bright fluorescent cells (indicative of apoptosis) were visible (left panel). However, following infection with AdWtp53 there were large numbers of brightly fluorescent cells (right panel) indicating the induction of an apoptotic pathway by wild type p53 transgene expression.

B. Nude Mice Studies.

In vivo effects of AdWtp53 were examined in human breast tumors MDA-MB-231 grown as xenografts in nude mice. 2 week old athymic mice (nu/nu) (Frederick Cancer Research Facility, Frederick, Maryland) were used in this study as an animal model for tumor growth. MDA-MB-231 cells were grown

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in monolayers. Prior to injections, cells were trypsinized, washed, and suspended in phosphate buffered saline. The mice were injected with 10^7 MDA-MB-231 cells in 0.1 PBS subcutaneously (5×10^6 cell/site) using a 21 g needle. Tumors were allowed to develop for 14 days. On day 14, tumors were either injected with AdWtp53 or AdControl (10^9 pfu/tumor) in the middle of the tumor using a 25G needle. An additional injection of either AdWtp53 or AdControl was administered on day 21 and the animals photographed on day 28. (See Figures 21A and 21B).

The results of this study indicate that those tumors of nude mice that were injected with AdWtp53 disappeared completely over the course of the treatment (See Figure 21A), while the tumors of the nude mice that were injected with AdControl (adenovirus only) increased in size to a final volume of 913 mm^3 in 21 days (See Figure 21B). Therefore, adenoviral vectors are useful for the eradication of cancer cells by contacting the cancer cells of the tumor with an amount of the adenoviral vector sufficient for the eradication of the cancer cells.

The results of this study also indicate that adenoviral vectors are useful in the prevention of the development of cancer cells in those subjects who are at risk of developing cancer. The preventative treatment involves the administration of an adenoviral vector expressing the desired DNA to a subject in an amount effective to prevent the development of cancerous cells.

EXAMPLE 25

Effect of AdWtp53 on Adriamycin and Mitoxantrone Resistant Human Breast Cancers.

To demonstrate that recombinant adenoviruses can be used to treat drug resistant cancers, cytotoxicity of AdWtp53 was studied in two human breast cancer MCF-7 cell lines which are resistant to adriamycin (MCF-Adr) and mitoxantrone (MCF-Mito).

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A. Cytotoxicity assays.

Cytotoxicity of adenovirus vectors was assessed after plating cells in 96 well plates (500 cells/well). The cells were incubated with various doses ($0-10^4$ pfu/cell) of adenovirus vectors for 7 days at 37°C . The cells were fixed with trichloroacetic acid and stained with 0.4% (wt/vol) sulforhodamine B (Sigma, St. Louis, MO) essentially as described previously (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). An O.D.₅₆₄ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and used as a measure of cell number.

B. Western blot analysis.

Cells (4×10^6) were plated in 15 cm tissue culture dishes and incubated with adenoviral vectors for 24 hours as described in Example 9. The cells were then scraped and cell lysates subjected to Western blot analysis as previously described (Katayose, et al., Clin. Cancer Res. (Submitted 1995)). The blots were probed with 3 $\mu\text{g/ml}$ of Ab-2 and Ab-6 for p53, 3 $\mu\text{g/ml}$ of EA 10 for WAF1/Cip1, with 3 $\mu\text{g/ml}$ of Actin (Ab-1) antibody. All antibodies were obtained from Oncogene Science (Uniondale, NY). The blots were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with horse radish peroxidase conjugated to secondary antibody and specific complex detected by the enhanced chemiluminescence technique according to the manufacturer's directions (Amersham, Arlington Heights, IL).

C. Cell Cycle Analysis.

Cells were plated in 6-well dishes (2×10^5 cells/well) and infected with adenoviral vectors (50 pfu/cell) for 48 hours. Cells were harvested by trypsinization and resuspended at a concentration of 2×10^5 cells/ml in 100% FBS and stored frozen until analyzed. Samples were stained for DNA cell cycle analysis using the previously described procedure. DNA content was measured using a FACScan flow cytometer (Becton-Dickenson, Mountain View, CA.). Cell cycle analysis of the resulting DNA histograms of cell

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number versus integrated red fluorescence was performed with Multicycle (Phoenix Flow Systems, San Diego, CA.) using a zero order polynomial to model the S-phase fraction. Debris, cell aggregates, and G0/G1 doublets were removed from the cell cycle analysis by software algorithms.

D. Nucleosomal DNA Fragmentation (Apoptosis) Analysis.

Cells (2×10^6) were plated in 15 cm dishes and the next day incubated with adenovirus vectors (50 pfu/cell) for 24 hours. Both adherent and floating cells were collected and pelleted by centrifugation at $1800 \times g$ for 5 minutes (RT-6000B, Du Pont, Boston). Low molecular weight DNA was prepared by a modified Hirt extraction method described previously, and evaluated on 2.5% agarose gel electrophoresis.

E. Cyclin Kinase Assays.

4×10^6 cells were plated in 15 cm dishes. The next day the cells were infected with different doses (1-200 pfu/cell) of recombinant adenoviruses for 24 hours. The cells were then harvested and lysed in a buffer. For cdk2 kinase activity, lysates were immunoprecipitated by anti-cdc2. For cdc2 kinase, cell lysates were immunoprecipitated. For cdc2-cyclin B-1 dependent kinase, lysates were precipitated with anti-cyclin B1. In brief, cell lysates were incubated with 1 ug primary antibody for 1 hour at 4°C . Immune complexes were collected on protein A-Sepharose beads. The beads were washed three times with EBC buffer and three times with kinase reaction buffer (20 mM Tris-HCL pH 7.5, 4 mM MgCl_2). The beads were then resuspended in kinase assay mixture containing 80 uM (^{32}P -ATP), histone H1 (2 ug) (Gibco-BRL). After incubation at 37°C , the reaction was stopped by the addition of 2X Laemli SDS sample buffer. Proteins were separated on 10% SDS-polyacrylamide gels, and the gels were then dried and autoradiographed.

The IC_{50} value for AdWtp53 in MCF-7 cells was 42 pfu/cell (See Table 5). However, in MCF-Adr and MCF-Mito

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cells, IC_{50} values were much lower (0.128 pfu and 2.4 pfu/cell, respectively.) These results suggest that MCF-Adr and MCF-Mito cells were much more sensitive to the cytotoxic effects of AdWtp53.

Table 5
AdWtp53 Cytotoxicity in Drug Resistant MCF-7 Cells

Cell line	p53 Status	AdWtp53 IC_{50} (pfu/cell)
MCF-7	Wild Type	42
MCF-Adr	Mutant	0.128
MCF-MITOXR	Wild Type	2.4

Western blot analysis of MCF-7, MCF-Adr and MCF-Mito following infection with various doses of AdWtp53 (1, 10 and 100 pfu/cell) showed high levels of p53 protein expression. In each cell line p53-expression was comparable and was dependent upon the dose of AdWtp53 used. However, a control adenovirus did not increase the p53 expression above the basal level. These results indicate that the increased sensitivity of drug resistant cells towards AdWtp53 was not due to higher transgene expression, but perhaps because of a distinct p53-mediated cascade in drug resistant cells.

F. AdWtp53-Mediated Cell Cycle Arrest.

To evaluate the mechanisms of AdWtp53-mediated cytotoxicity, the effects of AdWtp53 on cell cycle and apoptosis were examined. As shown in Figure 24, each cell line has a certain percent distribution of cells in G1, S and G2/M phase (Panel E,F,G). Following infection of cells with increasing doses of AdWtp53 (1-100 pfu/cell) for 24 hours, a dose-dependent reduction in cell population in S phase (shown as hatched curve) was observed in parental MCF-7 (Panel A-C) as well as the MCF-Adr (Panel D-F) and MCF-Mito (Panel G-I) cell lines. In addition to a decline in S-phase cells, in each cell line, accumulation of cells was observed in G1 phase (Figure 37) indicating a p53 induced

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G1/S arrest. In these studies a control adenovirus did not have any significant effect on the cell cycle pattern in any of the cell lines.

Although AdWtp53-mediated growth arrest was observed in all cell lines, there were some striking differences between MCF-7 and the drug resistant cells. In MCF-ADR and MCF-Mito cells, following infection with AdWtp53, there appeared to be a population of cells in sub G0 phase where apoptotic cells should accumulate. This accumulation of apoptotic cells in MCF-Adr and MCF-Mito increased with an increase in the dose of AdWtp53 used.

G. Effects of p53 Overexpression on cdk2 and cdc2-Cyclin B1 kinase.

Since the cell cycle arrest is likely to be related to the cyclin kinase activities, the effects of p53 overexpression on two cyclin kinases was examined. All the cell lines showed a basal level of cdk2 kinase. Following infection with AdWtp53 (1 pfu/cell) had little effect on the kinase activity, however, when a dose of 10 pfu/cell or greater was used, cdk2 kinase activity was significantly inhibited in all the cells examined. However, infection by a control adenovirus, cdk2 kinase activity was not lowered even at higher doses.

cdc2 kinase (cyclin B1 associated) was also expressed in all cell lines at a certain basal level. Following infection of these cells with a control adenovirus, the basal level of the enzyme activity was not reduced. In parental MCF-7 cells, cdc2 kinase was not much affected following AdWtp53 infection. However, infection of MCF-Adr with AdWtp53 resulted in complete inhibition of cdc2 cyclin B1-associated activity. Similar results were obtained with MCF-Mito.

H. AdWtp53-Mediated Apoptosis.

The effect of AdWtp53 infection on apoptosis was further examined in MCF-7 and the drug resistant cells by evaluating the nucleosomal DNA degradation, as described in Example 17.

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While in MCF-7 cells, AdWtp53 infection (100 pfu/cell) failed to induce any nucleosomal DNA degradation, both the drug resistant cells showed DNA laddering following 24 hour infection with AdWtp53. Even increasing the concentration of AdWtp53 up to 500 pfu/cell or increasing the length of incubation with AdWtp53 up to 48 hours did not induce any specific DNA laddering in the parental MCF-7 cells. Control adenovirus (100 pfu/cell) did not induce apoptosis in any of these cells. These results indicate that induction of apoptotic pathway may play an important role in determining the overall cytotoxicity of AdWtp53.

EXAMPLE 25

Consequence of p53 Gene Expression by Adenovirus Vector on Cell Cycle Arrest and Apoptosis in Human Aortic Vascular Smooth Muscle Cells.

A. Cytotoxicity Assays.

Cytotoxicity of adenoviral vectors was assessed in 96 well plates as described previously (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897). Briefly, cells (250 cells/well) were incubated with various doses (0-14⁴ pfu/cell) of adenovirus vectors, and cell number assessed after 7 days.

The amount of virus required to kill 50% cells (IC50) of AdWtp53 was 4 pfu/cell, whereas those of AdWAF1 and AdControl were 800 pfu/cell and 1200 pfu/cell, respectively. These results indicate that AdWtp53 was 200 and 300 times more toxic to AoVSMC than AdWAF1 and AdControl, respectively. This data is compatible with earlier results showing the existence of G1 subgroup in AoVSMC infected with AdWtp53. To determine whether AdWtp53-mediated cell death of AoVSCM occurred by apoptosis, DNA fragmentation analysis was performed. DNA fragmentation in AoVSMC infected with AdWtp53 was not detected. One possible explanation for this is that the number of cells susceptible to apoptosis might be too small in the beginning to detect DNA fragmentation

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because most AoVSMC in this experiment stayed in G1 phase. Another possibility is that the cytotoxicity of p53 to AoVSMC was induced by atypical apoptosis without DNA fragmentation (Oberhammer, F., et al. (1993) EMBO J. 12:3679-3684).

B. Western Blot Analysis.

Cells (1×10^6) were incubated with adenovirus vector for 48 hours. Cell lysates were subjected to Western blot analysis (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897). Blots were probed with 3 ug/ml of antibodies against p53 (Ab-2), p21 (EA1) and actin (AB-1) (Oncogene Science, Uniondale, NJ.) and analyzed using enhanced chemiluminescence technique (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897).

AoVSMC infected with AdWtp53 (50 pfu/cell) showed high levels of p53 expression, whereas AoVSMC infected with AdWAF1, AdControl (50 pfu/cell) or uninfected cells showed the low base level. Importantly, AdWtp53 induced p21 expression probably by direct transcriptional transactivation of the p21 gene. AoVSMC infected with AdWAF1 of 50 pfu/cell had much higher levels of p21 expressed compared to cells infected with AdControl or uninfected cells. This level of p21 expression is similar to p21 expressed by AdWtp53. AdControl slightly increased p21 protein levels as compared to uninfected cells. The protein levels of whole actin were unchanged in AoVSMC infected by AdWtp53, AdWAF1, AdControl, or uninfected cells.

C. Cell Cycle Analysis.

Cell cycle analysis was performed as described previously (Katayose, D. et al, in press, Cell Growth and Differ. (1995). Briefly, 2×10^5 cells were infected with adenoviral vectors (50 pfu/cell) for 48 hours. DNA content was measured using a FACSscan flow cytometer (Becton-Dickenson, Mountain View, CA.) Cell cycle analysis of the resulting DNA histograms of cell number versus integrated red fluorescence was performed with multi cycle (Phoenix

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Flow Systems, San Diego, CA) using a zero order polynomial to model the S-phase fraction.

AdControl had no effect on the cell cycle distribution compared to uninfected cells. By contrast, infection with either AdWTp53 or AdWAF1 (50 pfu/cell) decreased the number of S phase cells in dose dependent manner. While both AdWTp53 and AdWAF1 infection decreased S-phase cells, differential effects by AdWTp53 and AdWAF1 were observed; AdWTp53 induced the accumulation of G2/M phase cells (Figure 37, D, G), while AdWAF1 resulted in an increased number of G1 phase cells (Figure 37, C, E). From these results, it can be determined that p53 overexpression induced both G1 and G2/M cell cycle arrest, whereas p21 overexpression arrested cells at G1/S boundary. Additionally, AdWTp53-infected cells (50 pfu/cell) showed the population in G1 subgroup, indicating that some population of cells infected with AdWTp53 underwent apoptosis. No G1 subgroup was observed in cells infected with either AdWAF1 or AdControl.

D. Nucleosomal DNA Fragmentation Analysis.

Cells (2×10^6) was infected with adenoviral vectors (50 pfu/cell) for 48 hours. Both adherent and floating cells were collected and pelleted by centrifugation at 1800 x g for 5 minutes (RT-6000B, Du Pont, Boston). Low molecular weight DNA was prepared and evaluated on 2.5% agarose gel electrophoresis (Katayose, D., et al., (1995) Clin. Cancer Res. 1:889-897).

EXAMPLE 26

Induction of Cell Cycle Arrest by Adp27 in Human Breast Cancer Cells at G1/S and G2/M Checkpoints.

A. Adp27-mediated p27 expression. AdWTp53-mediated p27 expression was examined in MDA-MG-231 and MCF-7 human breast cancer cells following infection with Adp27. Western blot analyses demonstrated that the level of p27 increased substantially (at least 20-fold) following Adp27 (100 pfu/cell) infection in each cell line (Figure 27, Top

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Panel). In these experiments, AdControl vector did not increase p27 expression; and no change in actin protein level was detected in any of the cells lines following infection with either AdControl or Adp27.

B. Effect of Adp27 on cell cycle arrest. The effects of Adp27 infection on the two major growth regulatory mechanisms, cell cycle and apoptosis was investigated.

The effects of p27 overexpression on DNA cell cycle histograms were studied using different doses of Adp27 (1, 10, 50, 200 pfu/cell) and the results obtained are shown in Table 6. Mock infected cells had a basal distribution of cells (G1-%, S %, G2.M %). At an moi of 1 pfu/cell, there appeared to be an increase in the percentage of cells in G1 phase to %, and a decrease in S phase cells to %, indicating p27-induced G1/S arrest described above. However, at higher concentrations of Adp27 (100 moi or greater), cell number in G1 appeared to decrease with a concomitant increase in the cell number in G2/M stage. Adp27 produced a similar dose-dependent effects on cell cycle in MCF-7 cells. These results indicate that lower amounts of p27 can induce a strong G1/S arrest in breast cancer cells while higher amounts of p27 can either reverse this arrest or has additional check points at G2.M.

C. Effect of p27 Adp27 infection on cdk2 kinase and cdc2 (cyclin B1 associated kinase) activity.

To further investigate the biochemical mechanism of p27-mediated cell cycle control, the effects of Adp27 infection were also examined on the two key kinase activities responsible for G1/S and G2/M transition, which are cdk2 kinase and cdc2 (cyclin B-1 dependent) kinase. MDA-MB-231 cells had a certain basal level of both cdk2 and cdc2(cyclinB1-associated). Following infection of cells with Adp27, a dose dependent inhibition of both the kinase activities was observed. However, a control adenovirus did not cause the inhibition of these enzyme activities; in fact a slight increase in the activities was observed.

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D. Effect of Adp27 on apoptosis.

To investigate if p27-mediated effects on cell cycle progression will induce the cells to undergo apoptosis, the effects of Adp27 infection on the nucleosomal DNA degradation were studied. Interestingly, even a high dose of Adp27 (200 pfu/cell) did not induce any detectable DNA laddering in MDA-MB-231 cells. However, under the same conditions, AdWTp53 infection (100 pfu/cell) led to a DNA laddering typically seen in apoptotic cells. These results therefore indicate that while p27 can inhibit the cell cycle progression at G1/S and G2/M stages, it fails to induce apoptosis.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANTS: PREM K. SETH AND KENNETH COWAN
- 5 (ii) TITLE OF INVENTION: METHODS OF PREPARATION
AND USE OF ADENOVIRAL VECTORS
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
10 (A) ADDRESSEE: MORGAN & FINNEGAN, L.L.P.
(B) STREET: 345 PARK AVENUE
(C) CITY: NEW YORK
(D) STATE: NEW YORK
(E) COUNTRY: USA
(F) ZIP: 10154
- (v) COMPUTER READABLE FORM:
15 (A) MEDIUM TYPE: FLOPPY DISK
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WORDPERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
20 (A) APPLICATION NUMBER: TO BE ASSIGNED
(B) FILING DATE: FEBRUARY 15, 1996
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/390,604
(B) FILING DATE: February 17, 1995
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
25 (A) NAME: William S. Feiler
(B) REGISTRATION NUMBER: 26,728
(C) REFERENCE/DOCKET NUMBER: 2026-4185PCT
- (ix) TELECOMMUNICATION INFORMATION:
30 (A) TELEPHONE: (212) 758-4800
(B) TELEFAX: (212) 751-6849
(C) TELEX: 421792

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
35 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

- 92 -

- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: No
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY:
- (B) CLONE:
- (ix) FEATURE:
- (A) NAME/KEY:
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 20 base pair cDNA
sequence comprising a portion of the
E1 nucleotide sequence.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
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- (2) INFORMATION FOR SEQ ID NO:2:
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- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: No
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
- (F) TISSUE TYPE:

-93-

- (G) CELL TYPE:
- (H) CELL LINE:
- (I) ORGANELLE:

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 20 base pair cDNA sequence comprising a portion of the E1 nucleotide sequence.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

10 CAAGGTTTGG CATAGAAACC 20

(2) INFORMATION FOR SEQ ID NO:3:

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- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: No

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:
- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (E) HAPLOTYPE:
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- (H) CELL LINE:
- (I) ORGANELLE:

(ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 18 base pair cDNA sequence comprising a portion of exon seven of the p53 nucleotide sequence.

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
GTTGGCTCTG ACTGTACC 18

(2) INFORMATION FOR SEQ ID NO:4:

- 5 (i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 10 (iii) HYPOTHETICAL: No
- (vi) ORIGINAL SOURCE:
(A) ORGANISM:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
15 (E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:
- (ix) FEATURE:
20 (A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: 21 base pair cDNA
sequence comprising a portion of
exon eight of the p53 nucleotide
sequence.

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTCCGTCCC AGTAGATTAC C 21

(2) INFORMATION FOR SEQ ID NO:5:

- 30 (i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 35 (iii) HYPOTHETICAL: No

-95-

- (vi) ORIGINAL SOURCE:
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(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:
- (ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: 21 base pair cDNA
sequence comprising a portion of the
nucleotide sequence of WAF1.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
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- (2) INFORMATION FOR SEQ ID NO:6:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: No
- (vi) ORIGINAL SOURCE:
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(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
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(I) ORGANELLE:
- (ix) FEATURE:
(A) NAME/KEY:
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION: 21 base pair cDNA
sequence comprising a portion of the

-96-

- nucleotide sequence of WAF1.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTGCCATCTG TTTACTTCTC A 21

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What is claimed is:

1. An adenoviral vector construct comprising:
 - a. an origin of replication;
 - b. a left inverted terminal repeat;
 - c. a nucleotide sequence of the adenoviral genome, said sequence containing a first Clal restriction enzyme site and a second Clal site at the 5' end of the adenoviral genome; and
 - d. a homologous recombination domain.
2. The adenoviral vector of claim 1 further comprising DNA encoding suitable regulatory elements so as to effect expression of the polypeptide in a suitable host cell.
3. A circular, closed vector of claim 2.
4. The adenoviral vector of claim 2 further comprising heterologous DNA encoding a protein.
5. The vector of claim 4, wherein said vector is eucaryotic DNA.
6. The vector of claim 4, wherein the polypeptide encoded by the DNA is selected from the group consisting of cytosine deaminase, Jun/Fos dominant negative mutant, NO-synthetase, p27, GADD 45, p16, p15, mdm2, Rb, BAX, IL2, GMCF, p-53 antisense, Her/Neu2 antisense, and Erb4 antisense.

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7. The vector of claim 4 designated AdWTp53 and having ATCC Accession No. 97064.
8. A method of constructing a vector by homologous recombination between a shuttle vector and Clal cut genomic DNA derived from claim 1.
9. The method of claim 8 wherein the shuttle vector is selected from the group consisting of pDK10, pDK13, pCG1, pPS1 and pCG2.
10. The adenoviral vector produced by the method of claim 8 further comprising DNA encoding suitable regulatory elements so as to effect expression of the polypeptide in a suitable host cell.
11. A circular, closed vector of claim 10.
12. The adenoviral vector of claim 10 further comprising heterologous DNA encoding a protein.
13. The vector of claim 10, wherein said vector is eucaryotic DNA.
14. The vector of claim 10, wherein the polypeptide encoded by the DNA is selected from the group consisting of NO-synthetase, GADD 45, p16, p15, mdm2, Rb, BAX, IL2, GMCF, p-53, p-53 antisense, Her/Neu2 antisense, and Erb4 antisense.

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15. A host cell containing the vector construct of claim 10.
16. The vector of claim 10 designated AdWAF1 and having ATCC Accession No. 97063.
- 5 17. The vector of claim 10 designated Adp27 and having the structure shown in Figure 25.
18. The vector of claim 10 designated AdCD and having the structure shown in Figure 29.
- 10 19. The vector of claim 10 designated Adp16 and having the structure shown in Figure 31.
- 15 20. The vector of claim 10 designated AdTAM67 and having the structure shown in Figure 33.
21. The vector of claim 10 designated AdB7-1 and having the structure shown in Figure 34.
- 20 22. The vector of claim 10 designated AdB7-2 and having the structure shown in Figure 35.
- 25 23. A method for inhibiting the proliferation of cells, comprising contacting the cells with an amount of the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 effective to inhibit cell proliferation.
- 30 24. The method of claim 23 wherein the cells are epithelial cells.

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25. The method of claim 24 wherein the epithelial cells are mammary epithelial cells or lung epithelial cells.
26. The method of claim 23 wherein the cells are cancer cells.
27. The method of claim 23 wherein the cancer cells are selected from the group consisting of human melanoma cells, human mammary tumor cells, human lung tumor cells, human sarcoma cells or carcinoma cells.
28. The method of claim 23 wherein the mammary tumor cells are selected from the group consisting of MDA-MB-231, MCF-7, MCF-Adr and MCF-Mito.
29. The method of claim 23 wherein the lung tumor cells are H-358 cells.
30. The method of claim 23 wherein the cancer cells are resistant to drugs.
31. A composition for inhibiting the proliferation of cells comprising the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 and a suitable carrier.
32. A method of treating a subject suffering from abnormal cell proliferation, comprising administering to the subject an amount of the

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composition of claim 31 effective to inhibit the abnormal cell proliferation.

33. The method of claim 32, further comprising administering the composition in conjunction with a chemotherapeutic agent.

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34. The method of claim 32, further comprising administering the composition in conjunction with irradiation treatment.

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35. The method of claim 32 wherein the abnormally proliferating cells comprise tumor cells.

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36. The method of claim 35 wherein the tumor cells are selected from the group consisting of human melanoma cells, human mammary tumor cells or human sarcoma cells.

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37. The method of claim 32 wherein the abnormal cell proliferation comprises abnormal vascularization.

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38. The method of claim 32, wherein the mode of administration of the composition is selected from the group consisting of intravenous, subcutaneous, intramuscular, intratumor or local.

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39. The method of claim 32, wherein the mode of administration of the composition effective to inhibit the abnormal cell proliferation is between about 10^6 and 10^8 plaque forming units per tumor.

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40. A method of treating a subject suffering from a tumor which comprises administering to the subject an effective amount of the composition of claim 31 effective to eradicate the tumor cells.
- 5 41. The method of claim 40, wherein the tumor cells are selected from the group consisting of human melanoma cells, human mammary tumor cells, human lung tumor cells, human sarcoma cells or carcinoma cells.
- 10 42. A method of treating a subject at risk of developing cancer which comprises administering to the subject an effective amount of the composition of claim 31 effective to prevent the development of cancerous cells.
- 15 43. A pharmaceutical composition comprising the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 in an amount effective to inhibit cell proliferation, and a pharmaceutically acceptable carrier.
- 20 44. A pharmaceutical composition for purging bone marrow cells or contaminating tumor or cancer cells comprising the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 in a therapeutically effective amount and a
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pharmaceutically acceptable carrier.

45. A method of purging bone marrow cells of contaminating cancer cells ex vivo by contacting the contaminating cells with an amount of the vector construct of claim 4, or claim 7, or claim 12, or claim 16, or claim 17, or claim 18, or claim 19, or claim 20, or claim 21, or claim 22 to effectively eradicate the contaminating cells.

46. The method of claim 45 further comprising re-introducing the purged bone marrow cells into a patient.

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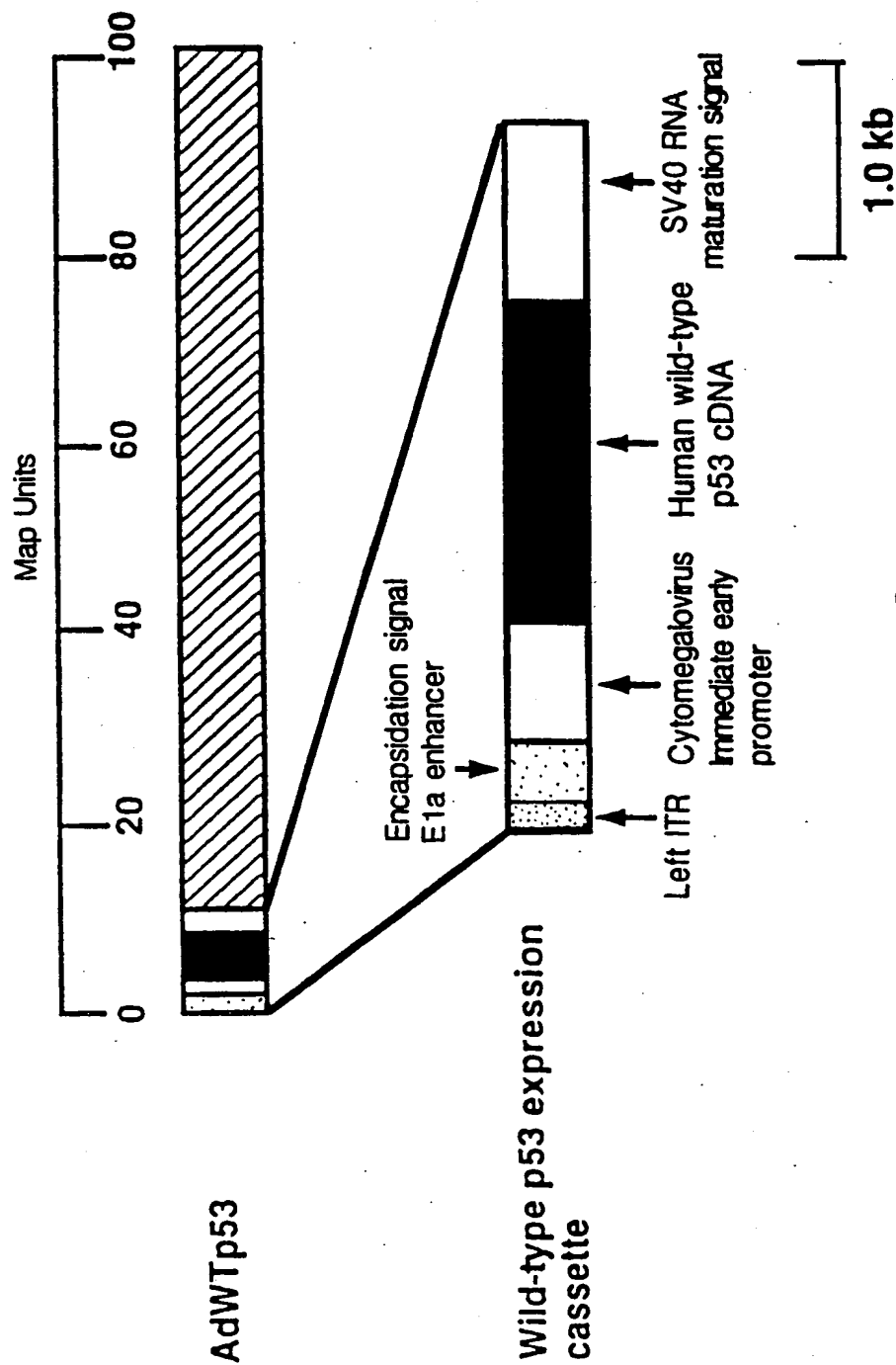


FIG.1

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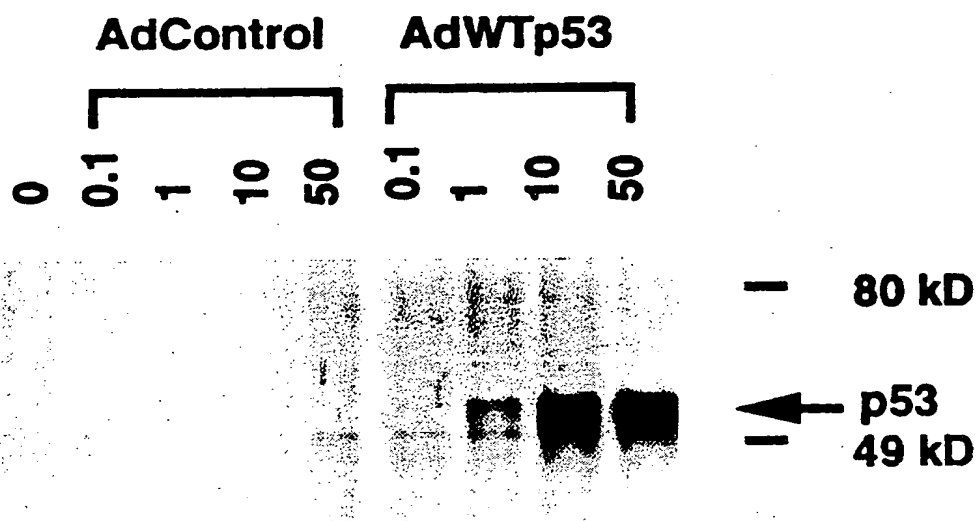


FIG. 2A

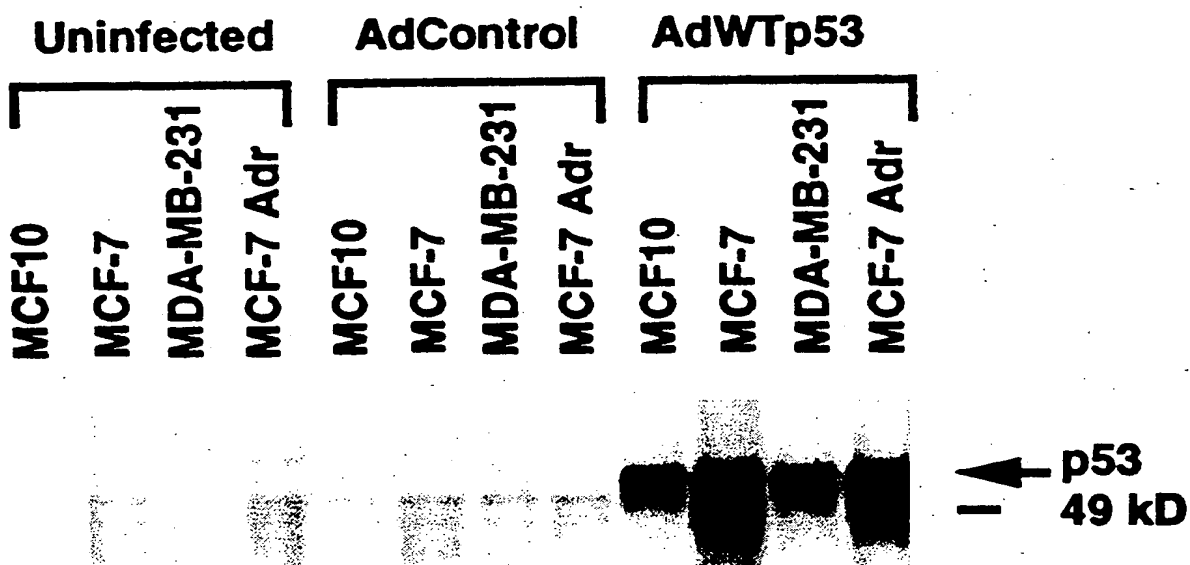


FIG. 2B

SUBSTITUTE SHEET (RULE 26)

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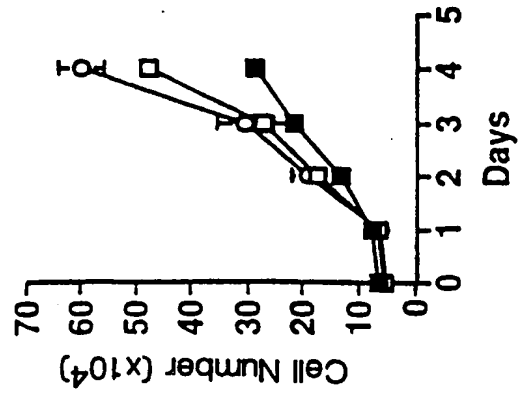


FIG.3C

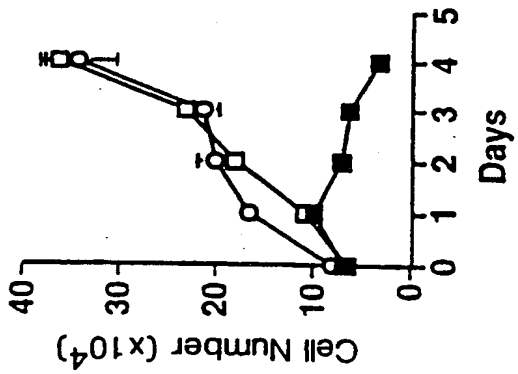


FIG.3B

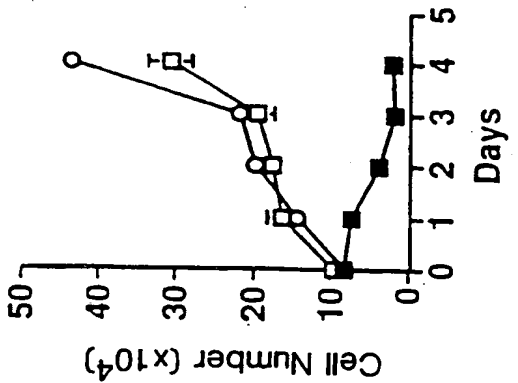
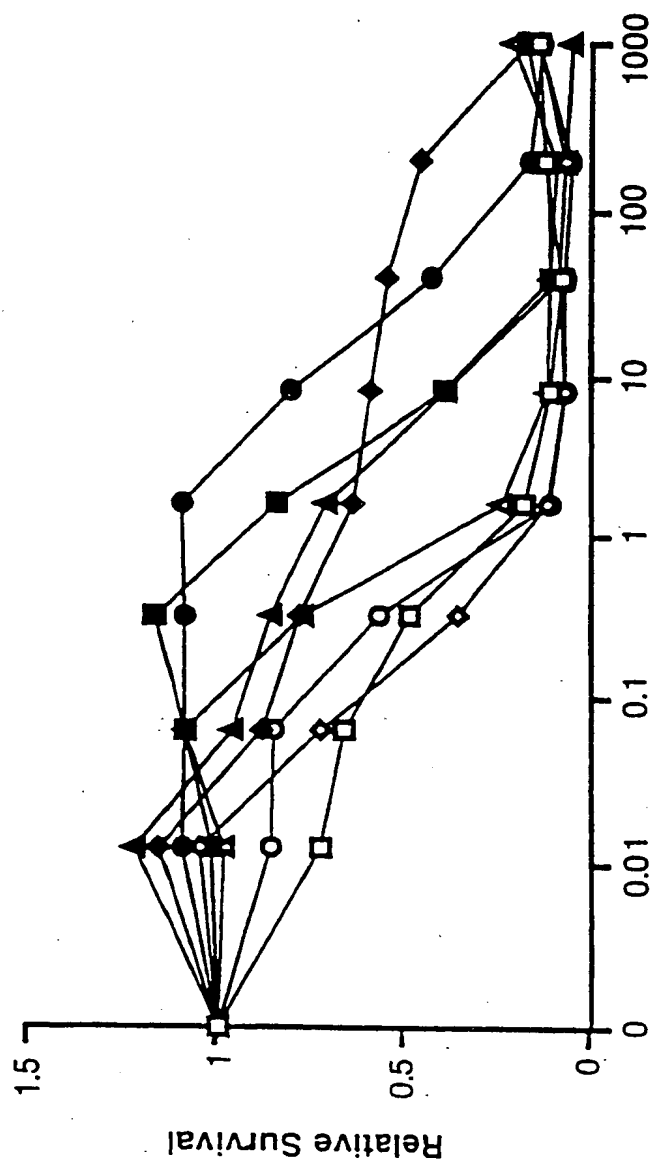


FIG.3A

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PFU/cell
FIG. 4

SUBSTITUTE SHEET (RULE 26)

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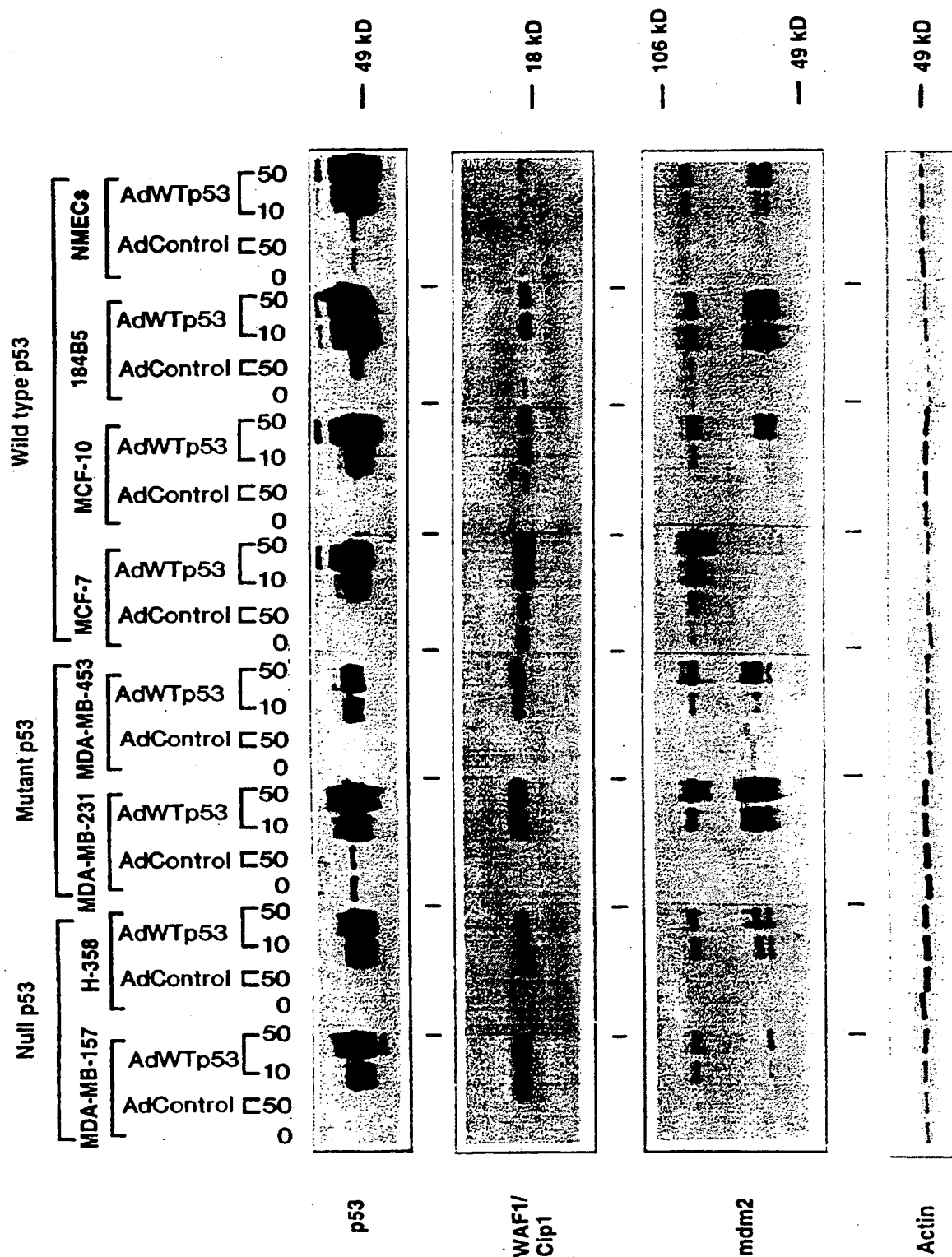


FIG. 5

SUBSTITUTE SHEET (RULE 26)

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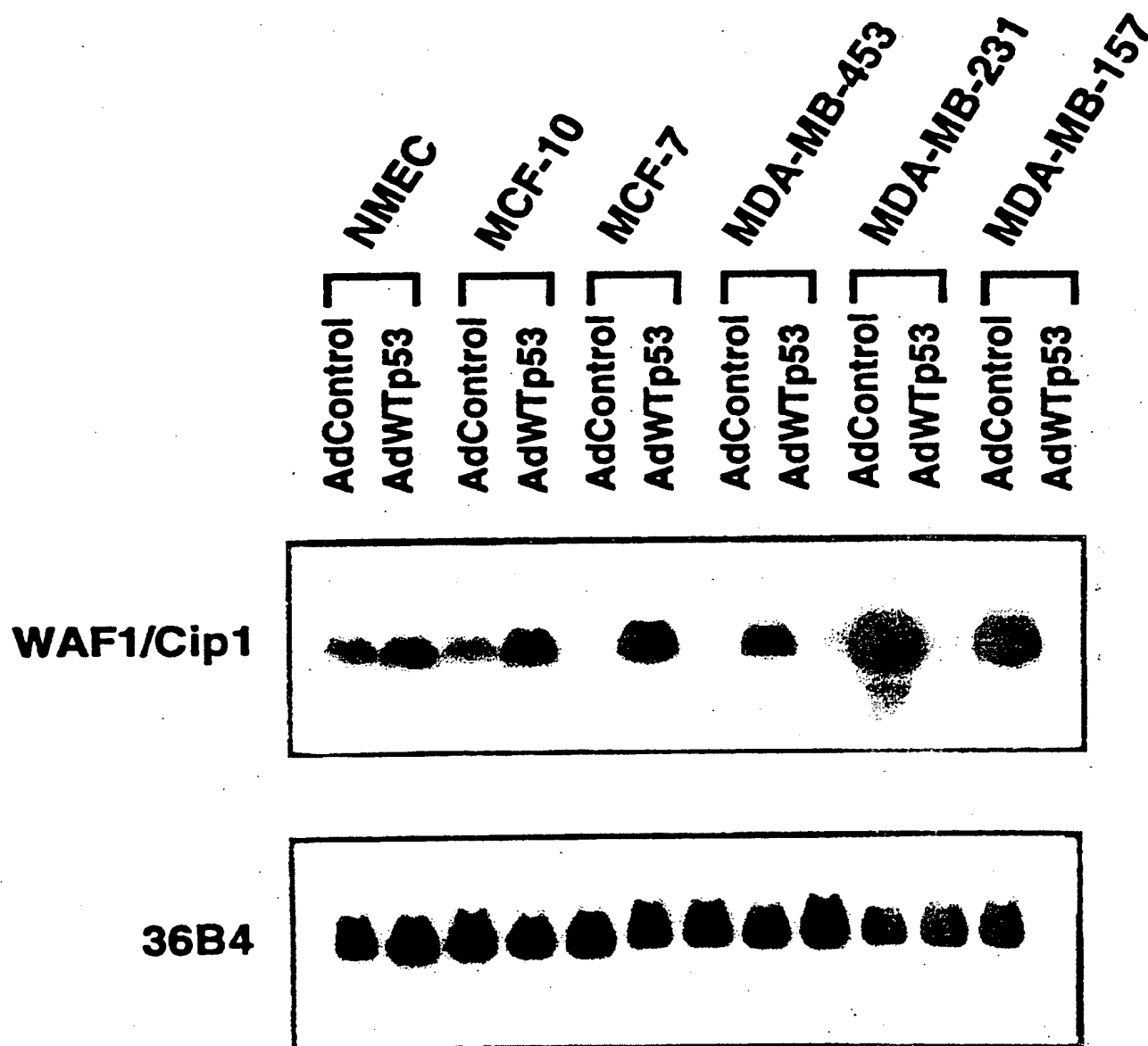


FIG. 6

SUBSTITUTE SHEET (RULE 26)

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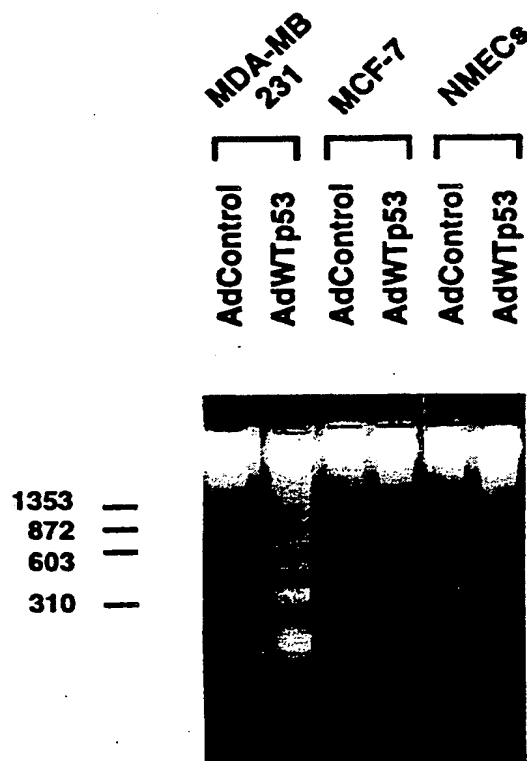


FIG. 7

SUBSTITUTE SHEET (RULE 26)

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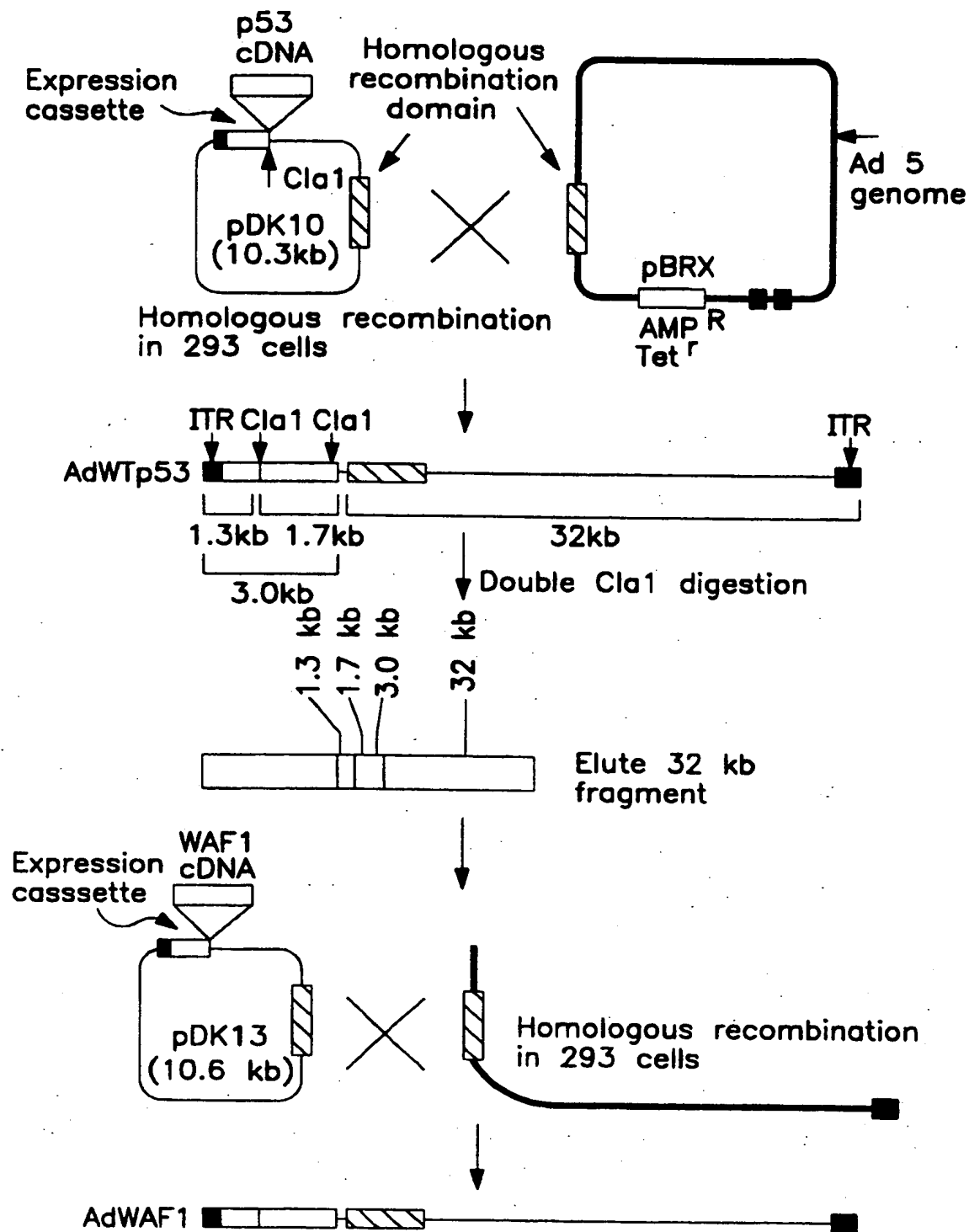


FIG. 8

SUBSTITUTE SHEET (RULE 26)

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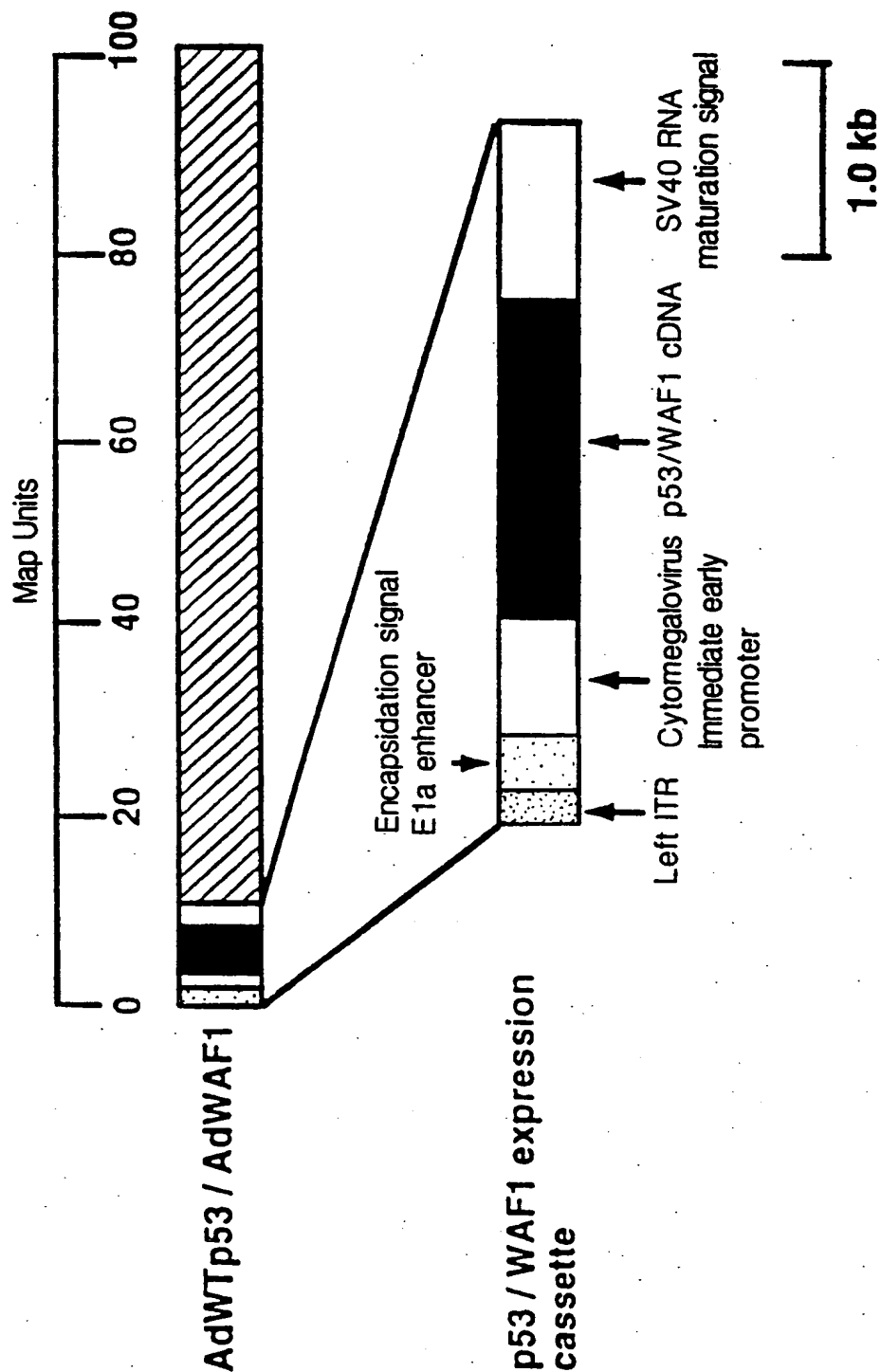


FIG.9

SUBSTITUTE SHEET (RULE 26)

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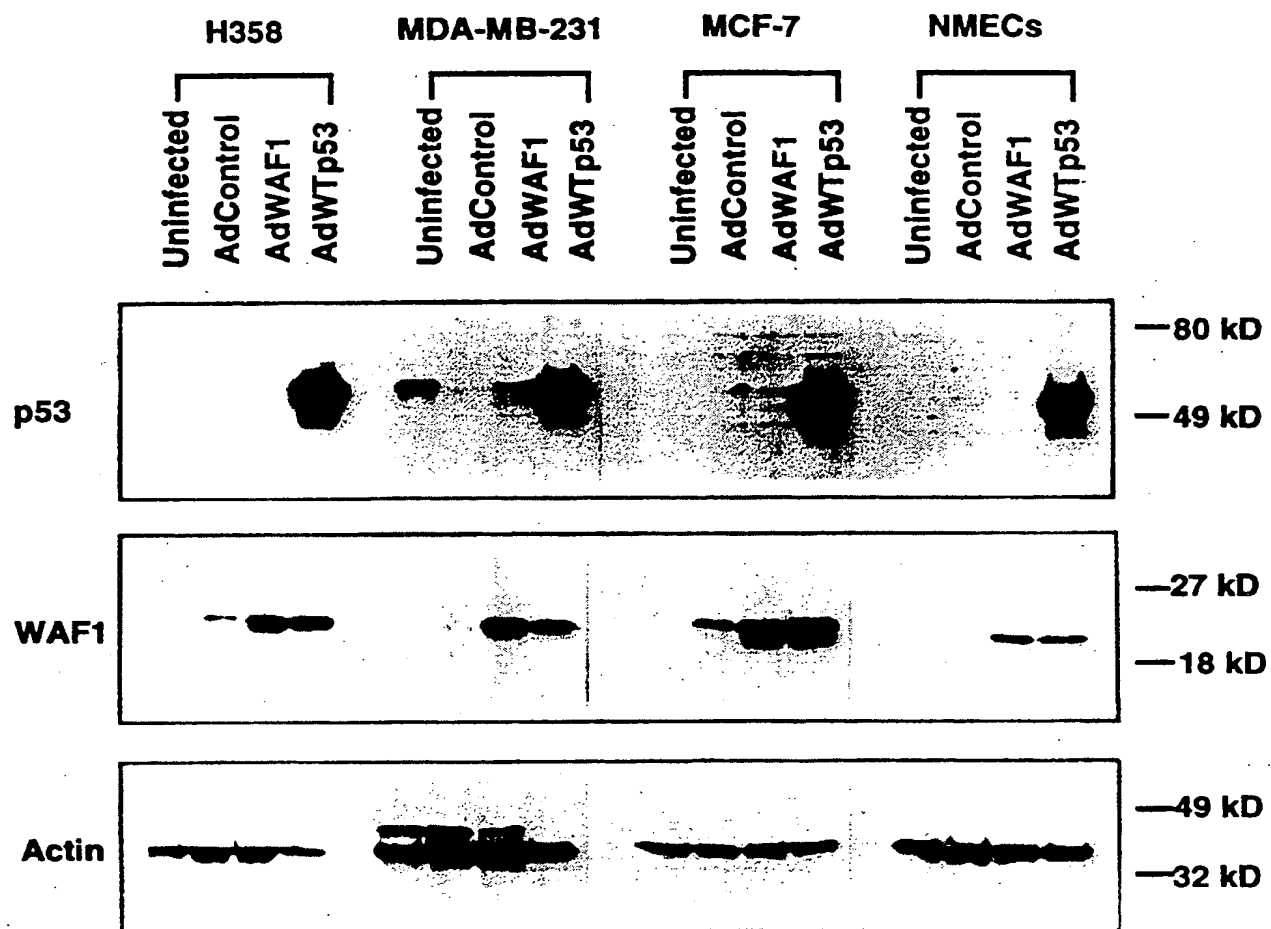


FIG. 10

SUBSTITUTE SHEET (RULE 26)

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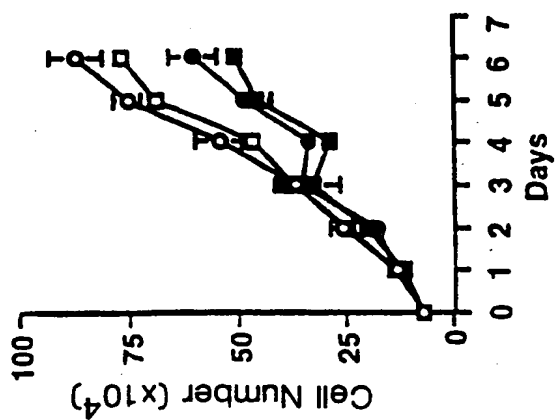


FIG. IIC

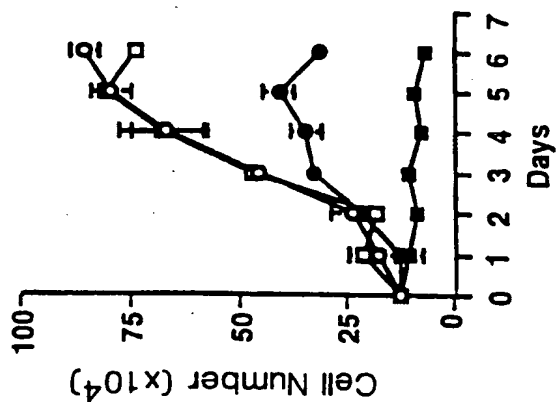


FIG. IIB

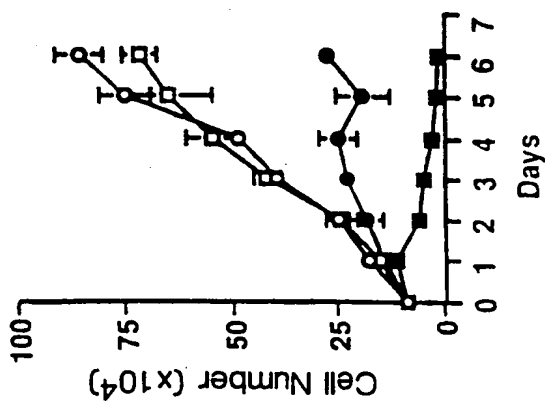


FIG. IIA

SUBSTITUTE SHEET (RULE 26)

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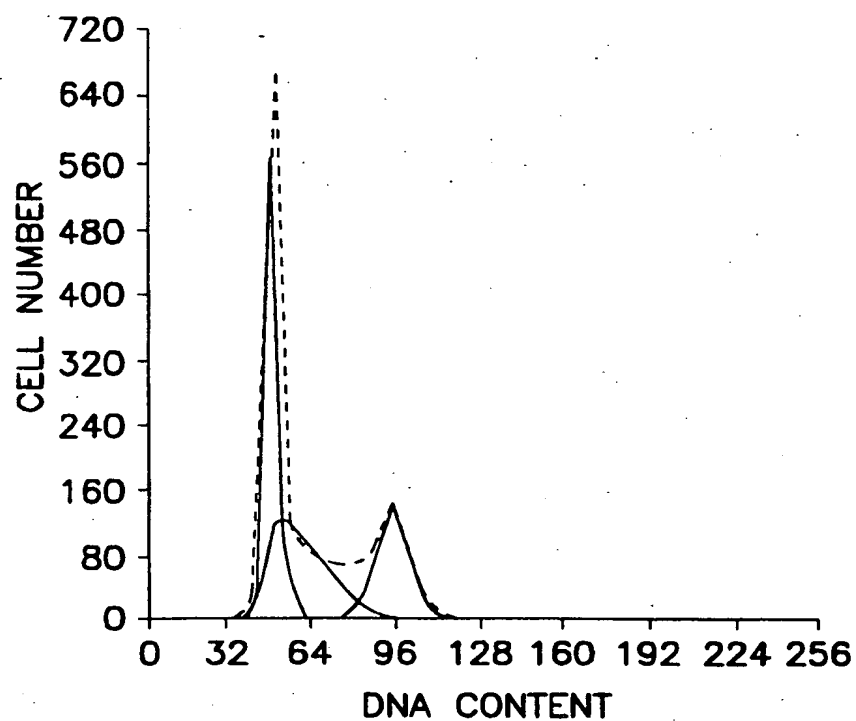


FIG. 12A

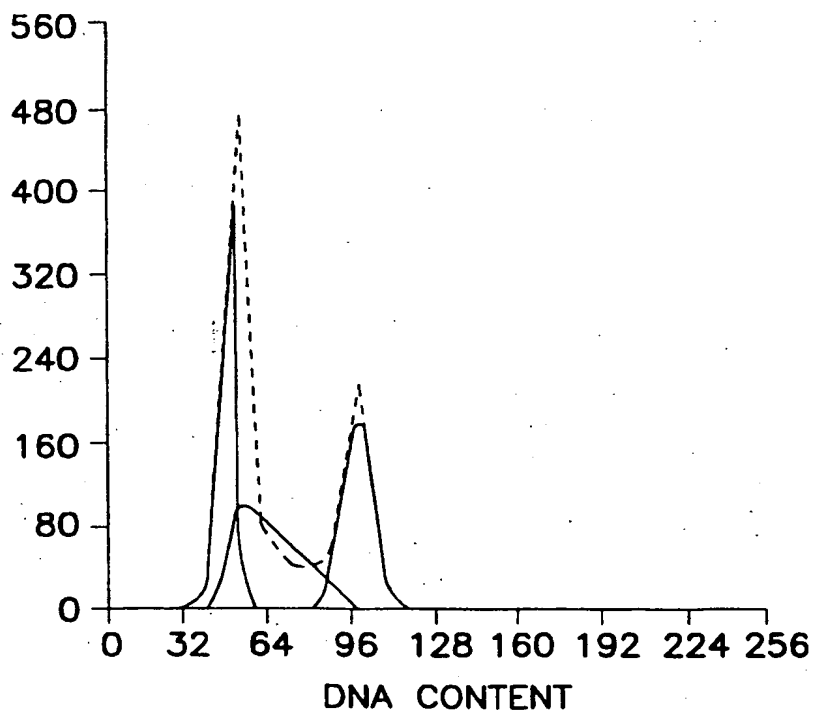


FIG. 12B

SUBSTITUTE SHEET (RULE 26)

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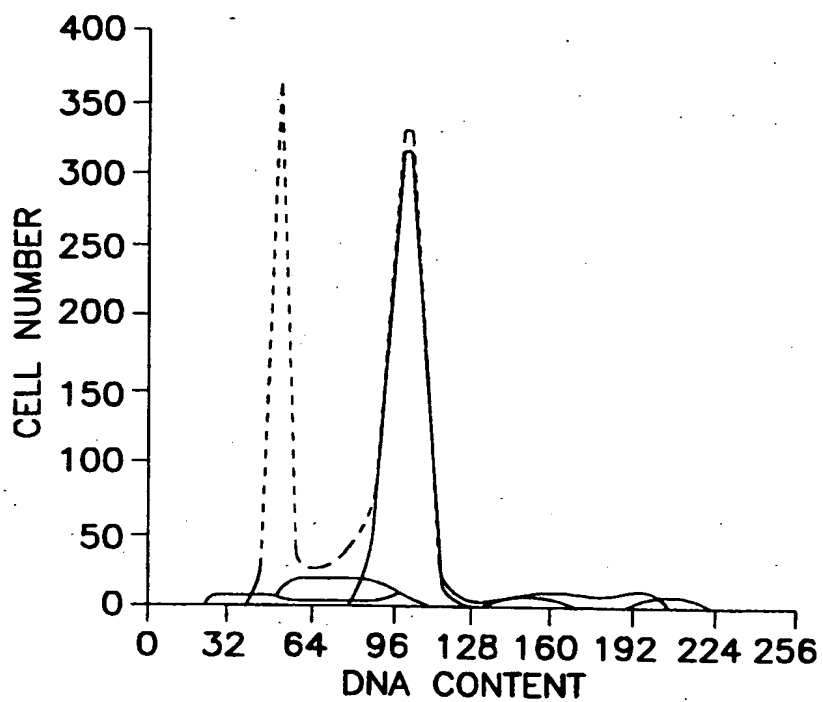


FIG. 12C

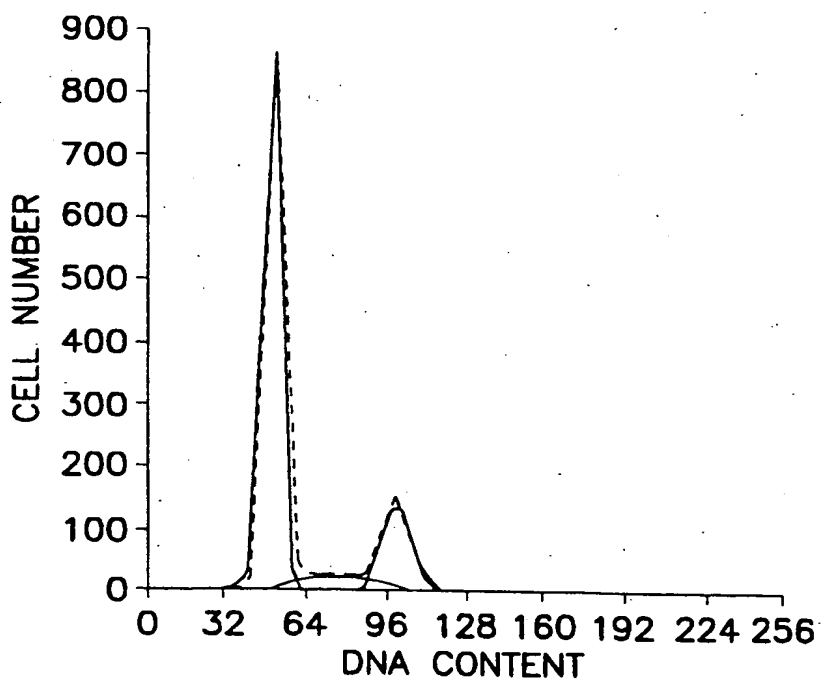


FIG. 12D

SUBSTITUTE SHEET (RULE 26)

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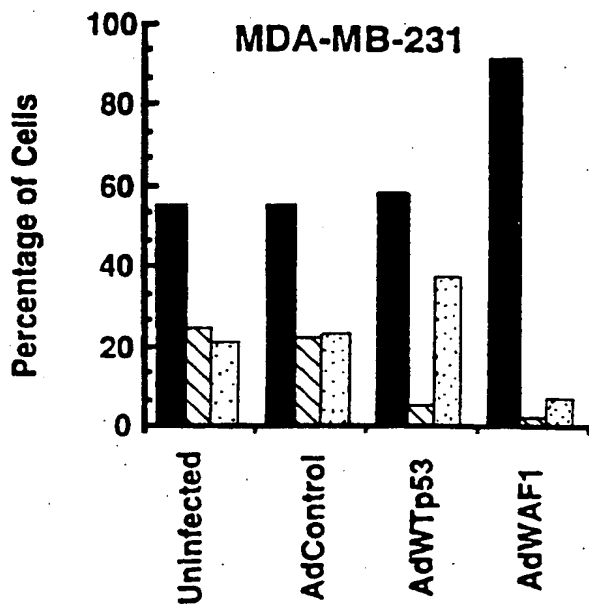


FIG. 13A

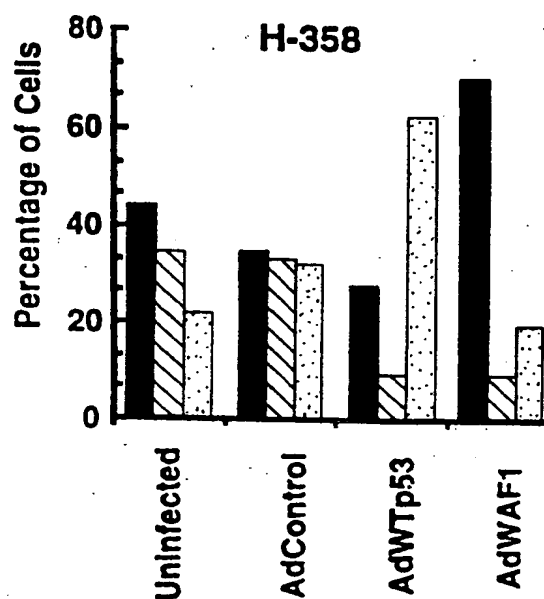


FIG. 13B

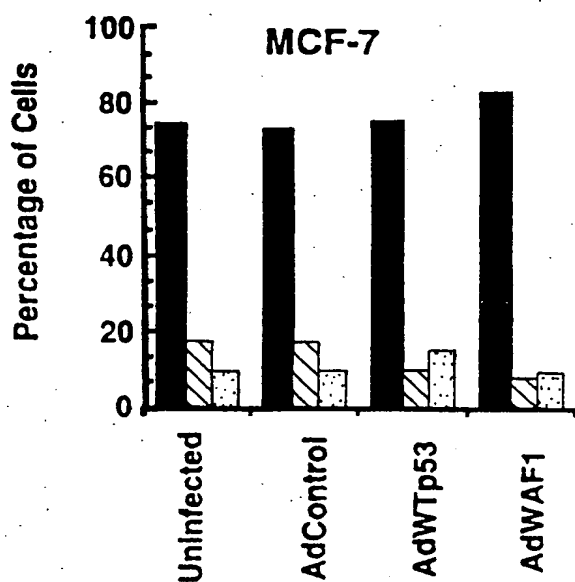


FIG. 13C

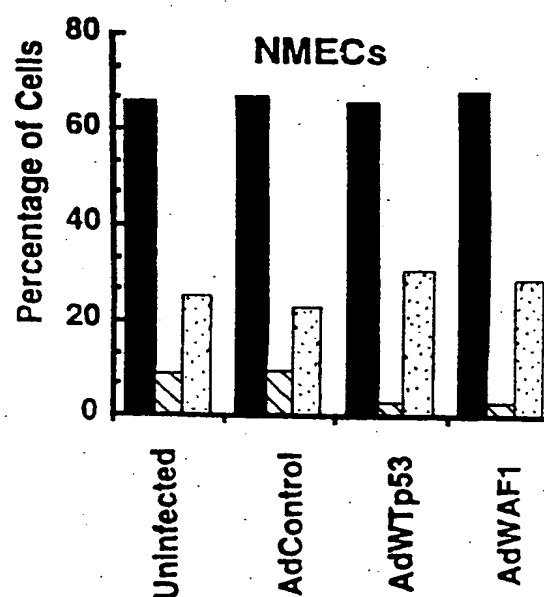


FIG. 13D

SUBSTITUTE SHEET (RULE 26)

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FIG. 14A

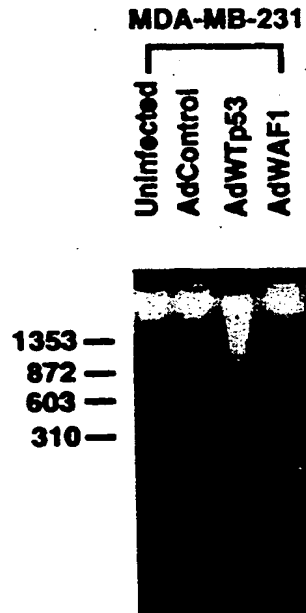


FIG. 14B

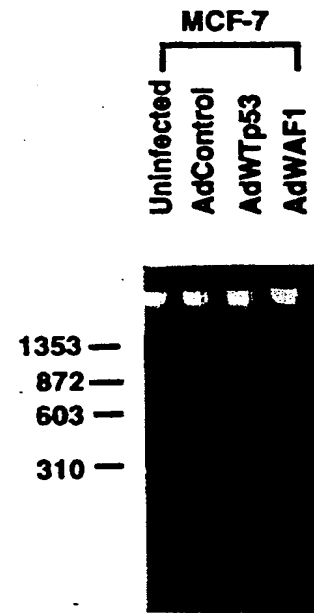


FIG. 14C

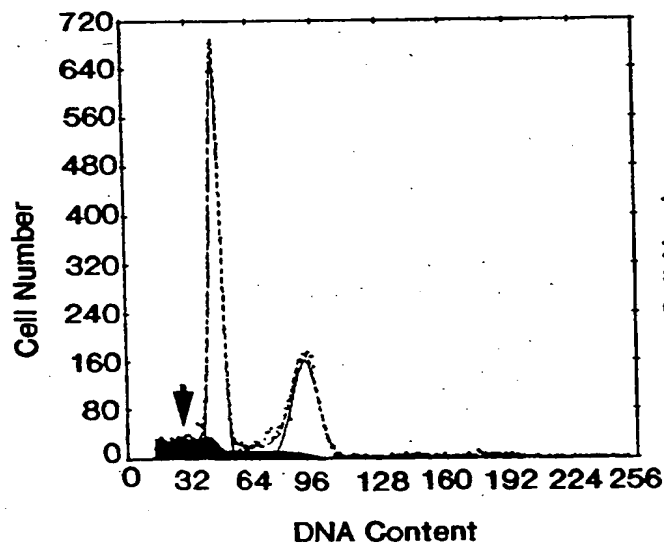


FIG. 14D

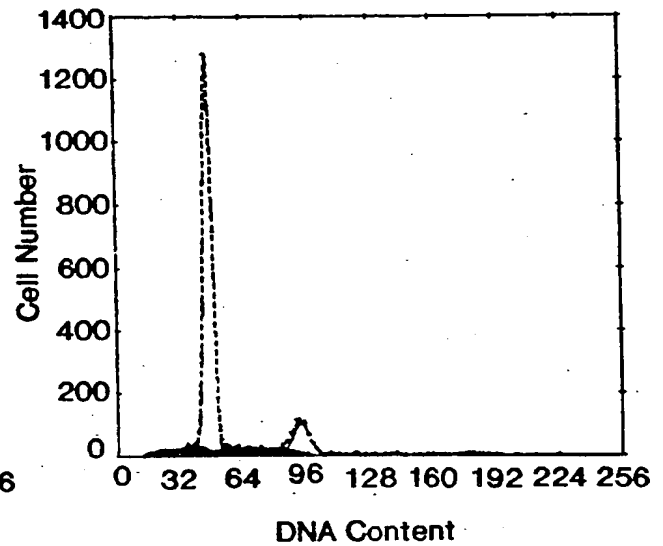


FIG. 14E

SUBSTITUTE SHEET (RULE 26)

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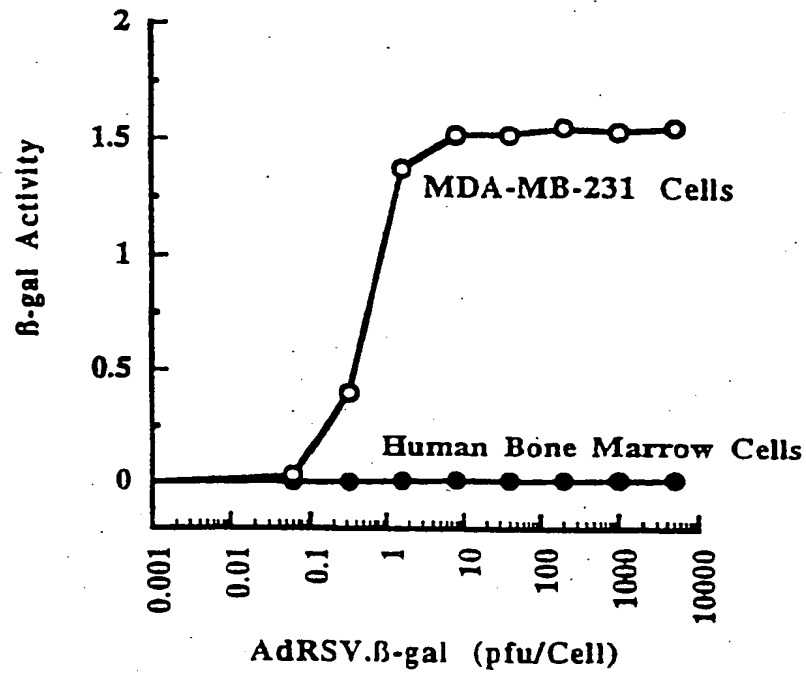


FIG. 15

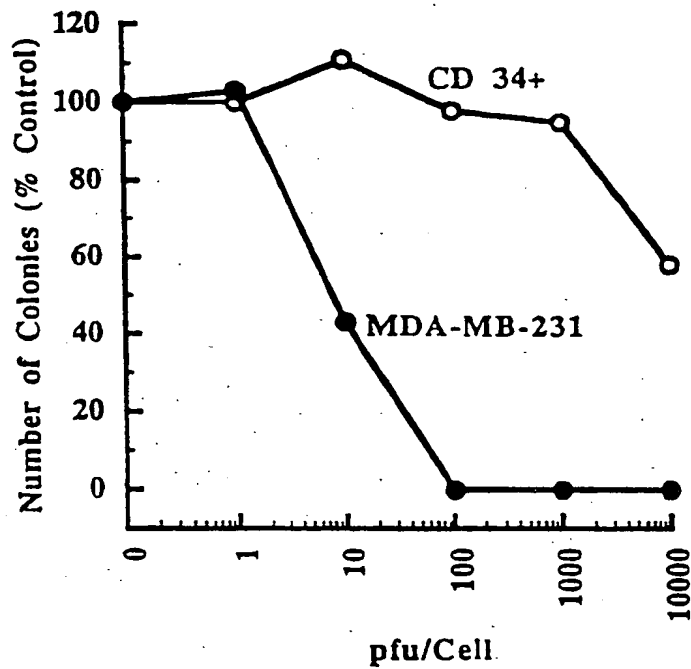


FIG. 16

SUBSTITUTE SHEET (RULE 26)

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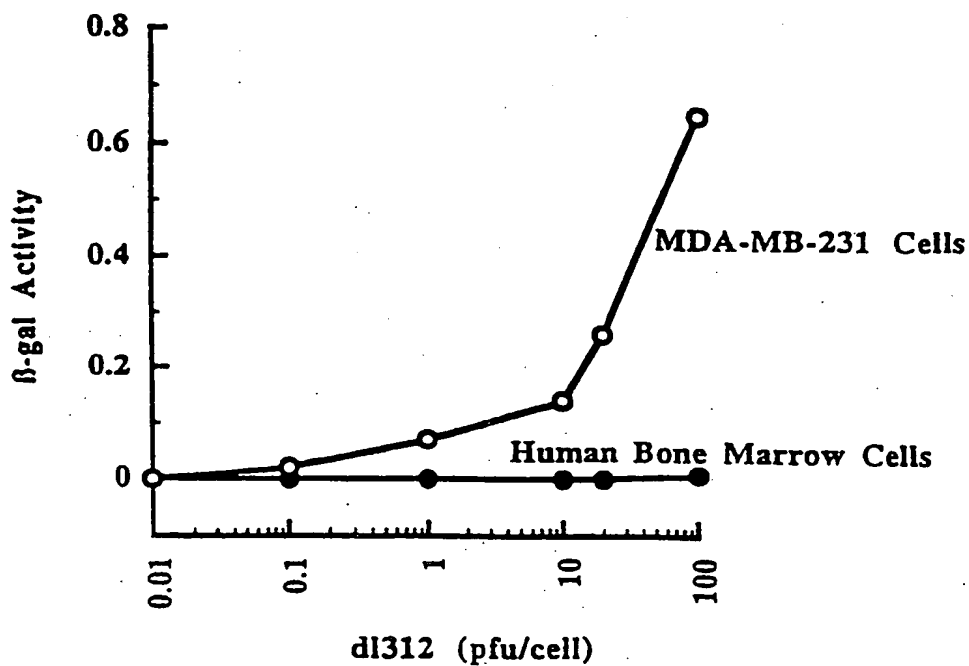


FIG. 17

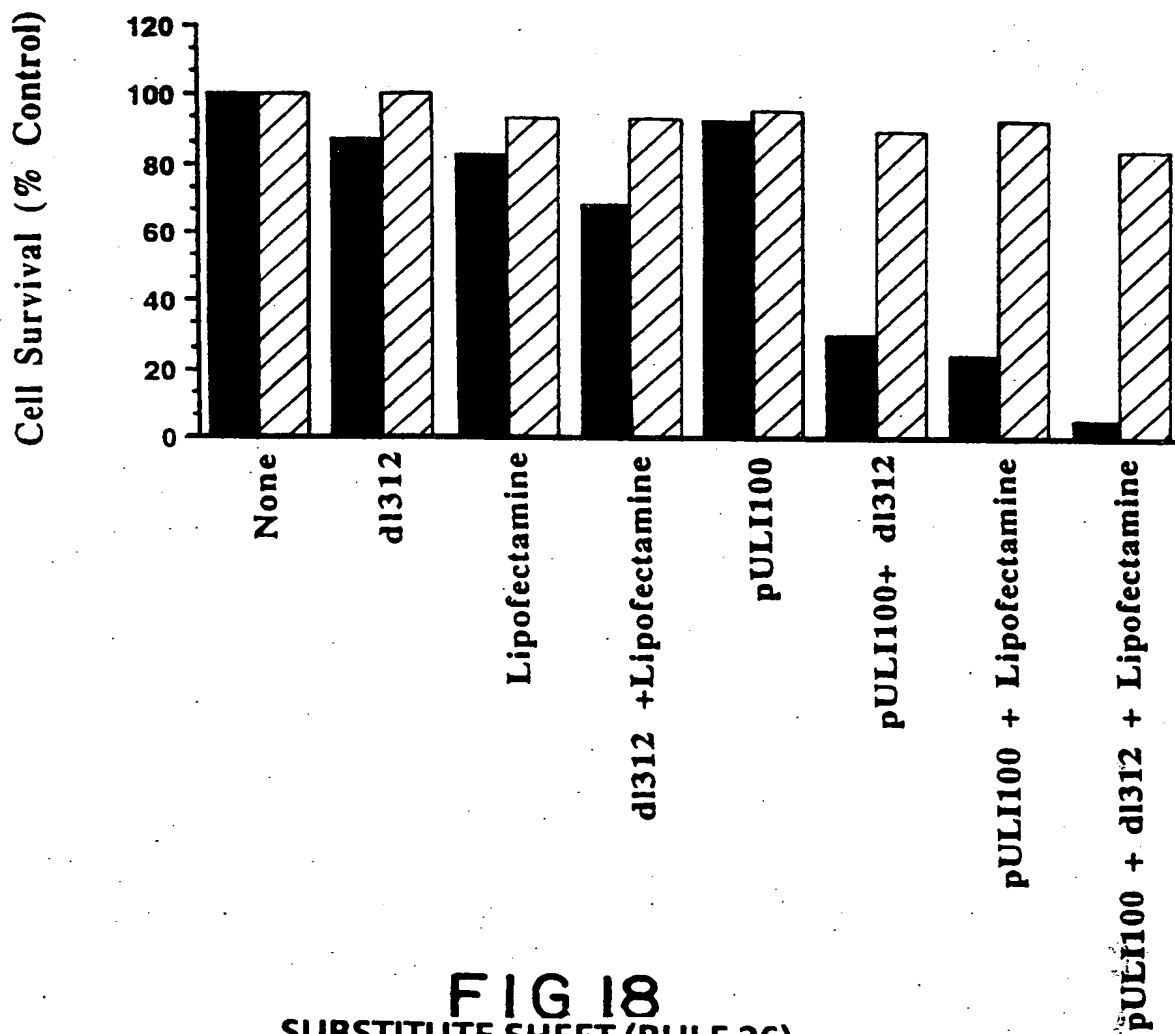


FIG 18

SUBSTITUTE SHEET (RULE 26)

AdControl

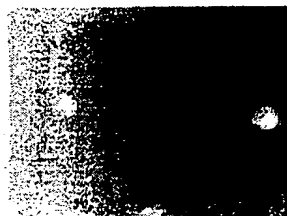


FIG. 19A

AdWTp53



FIG. 19B

SUBSTITUTE SHEET (RULE 26)

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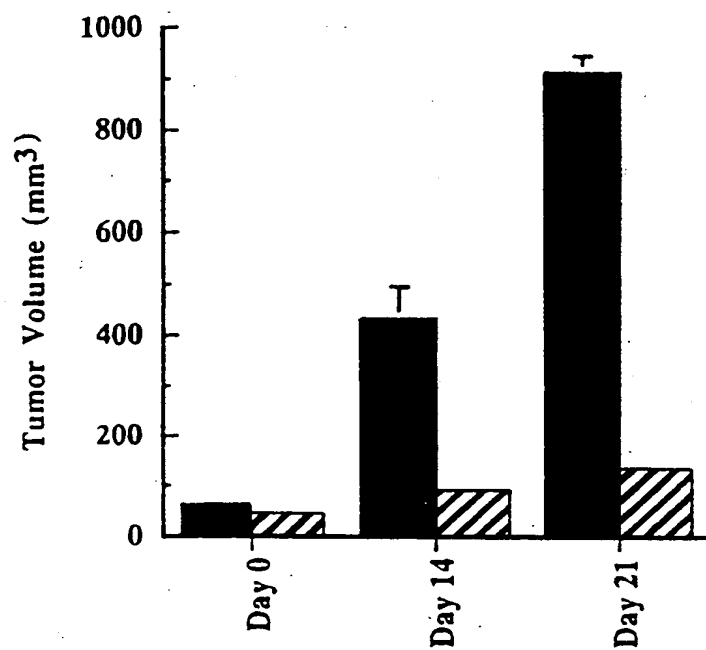


FIG. 20

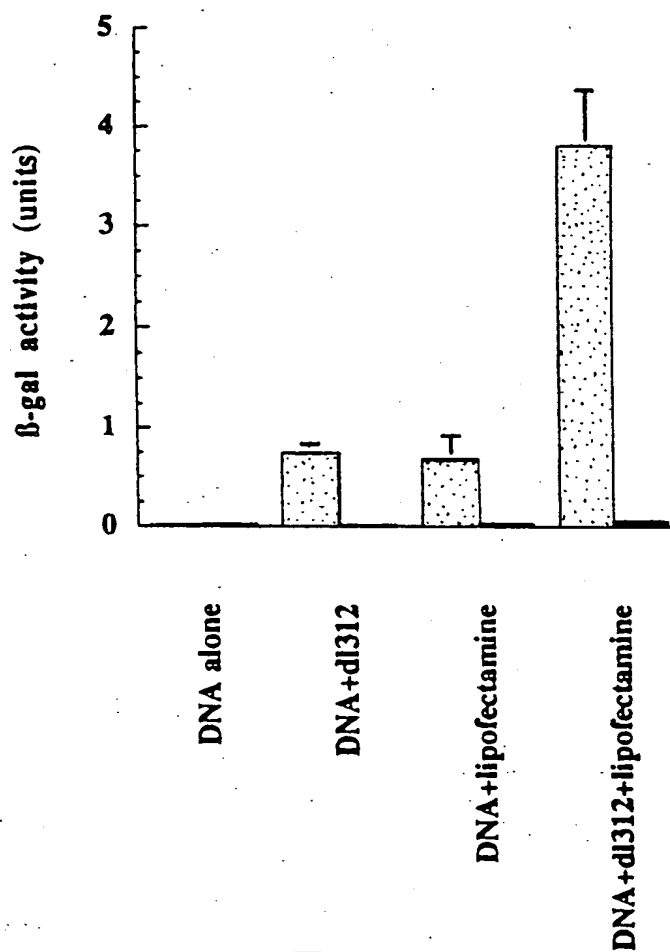


FIG. 22

SUBSTITUTE SHEET (RULE 26)

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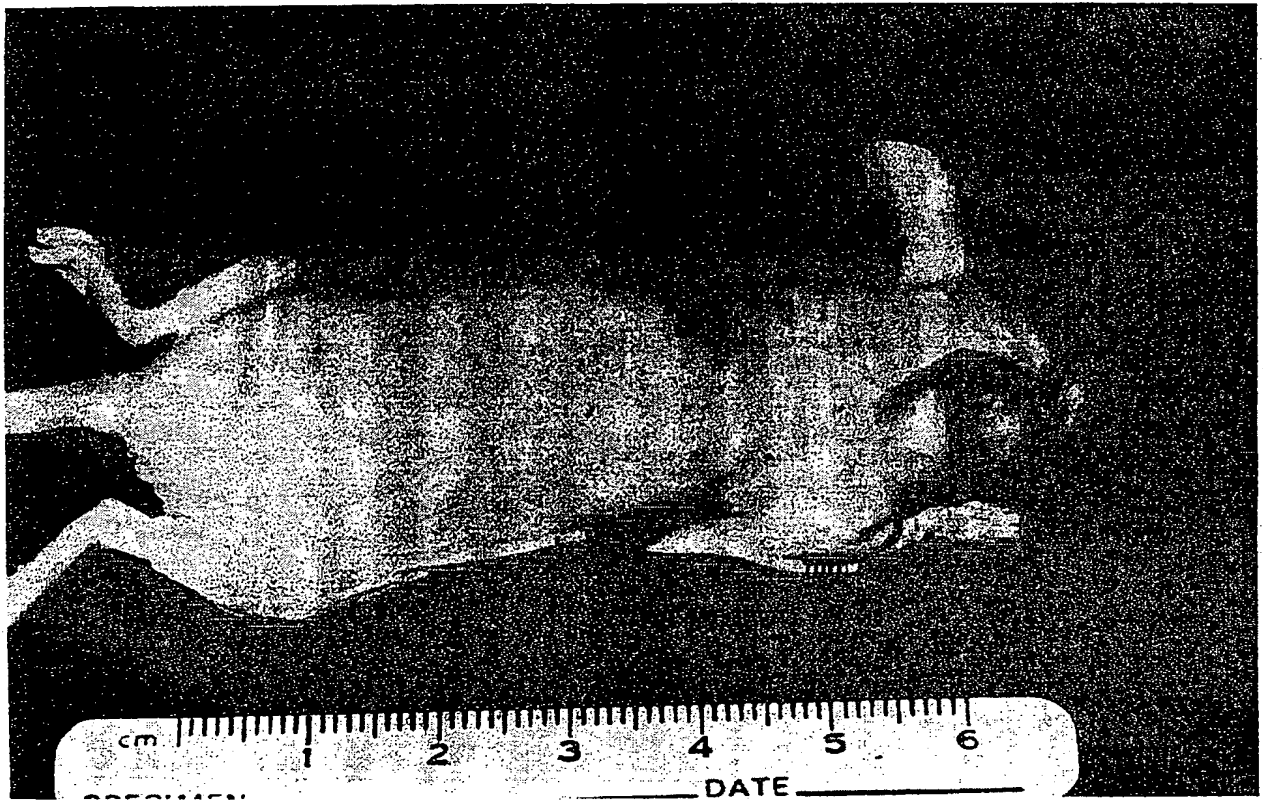


FIG. 2IA

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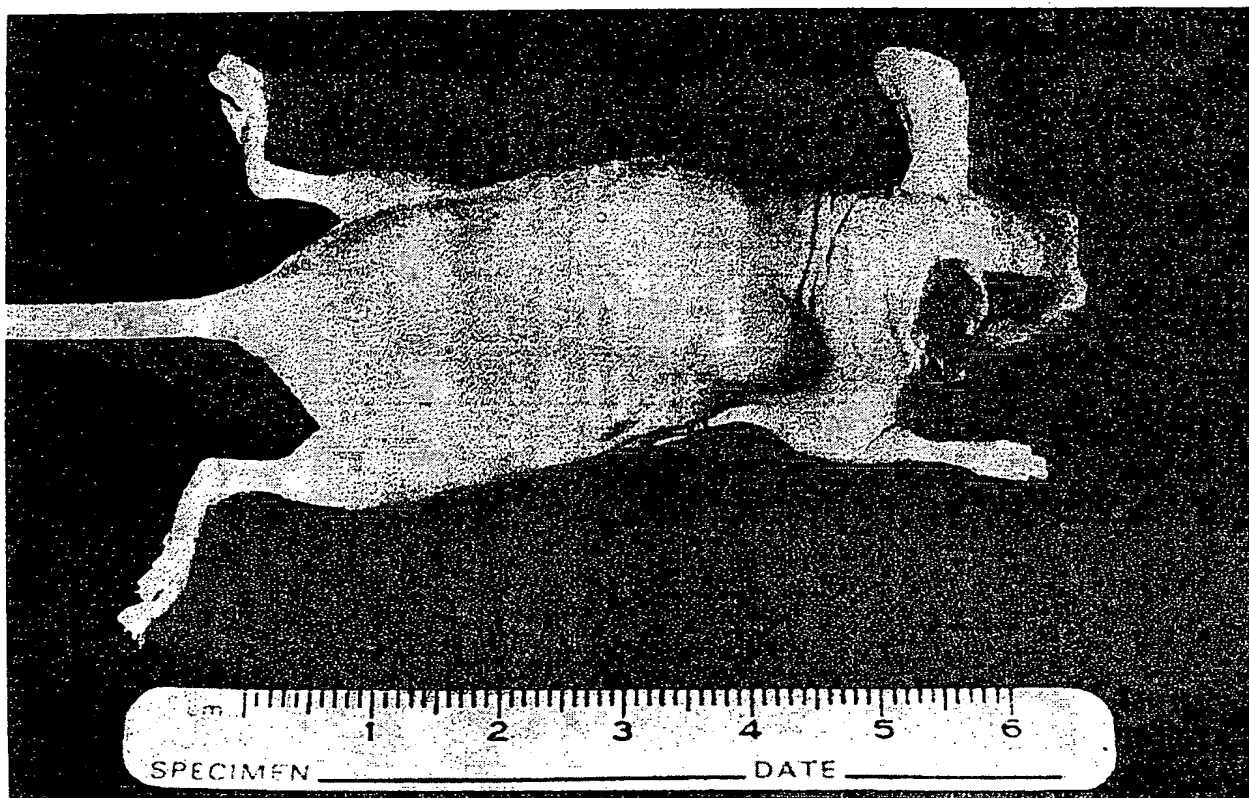


FIG. 21B

SUBSTITUTE SHEET (RULE 26)

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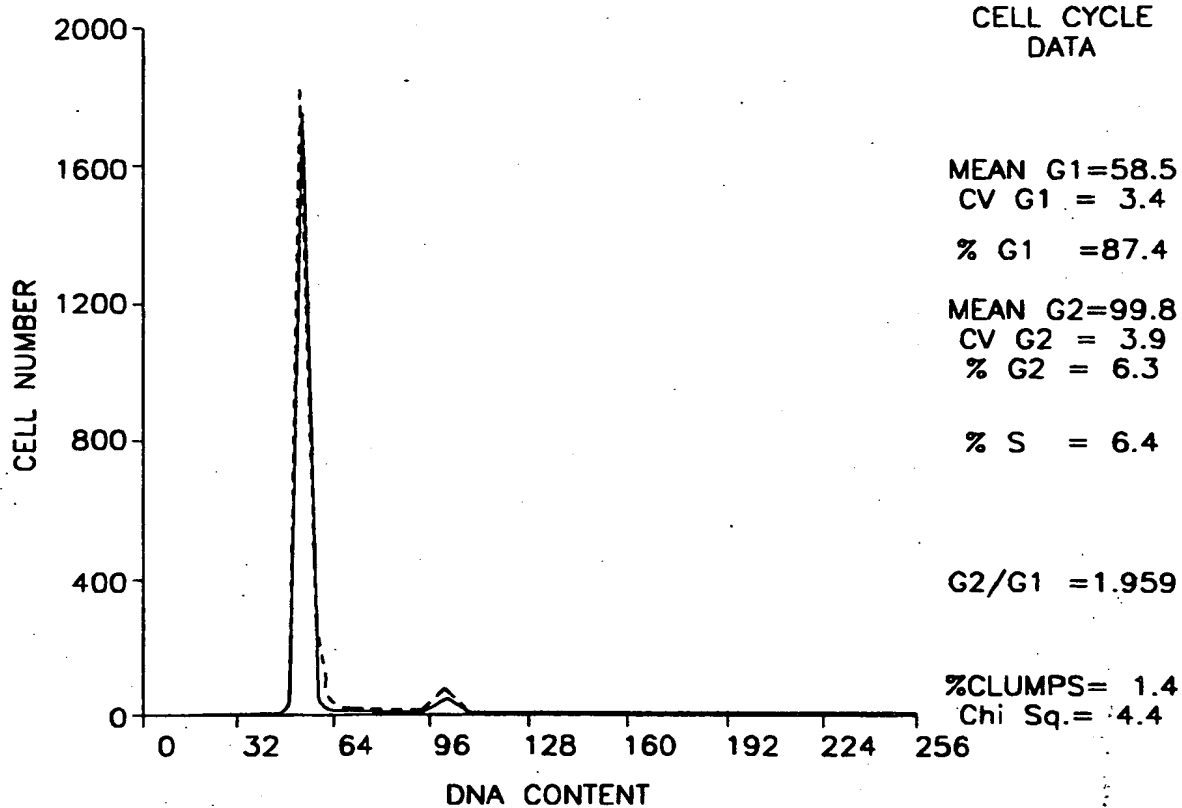


FIG. 23A

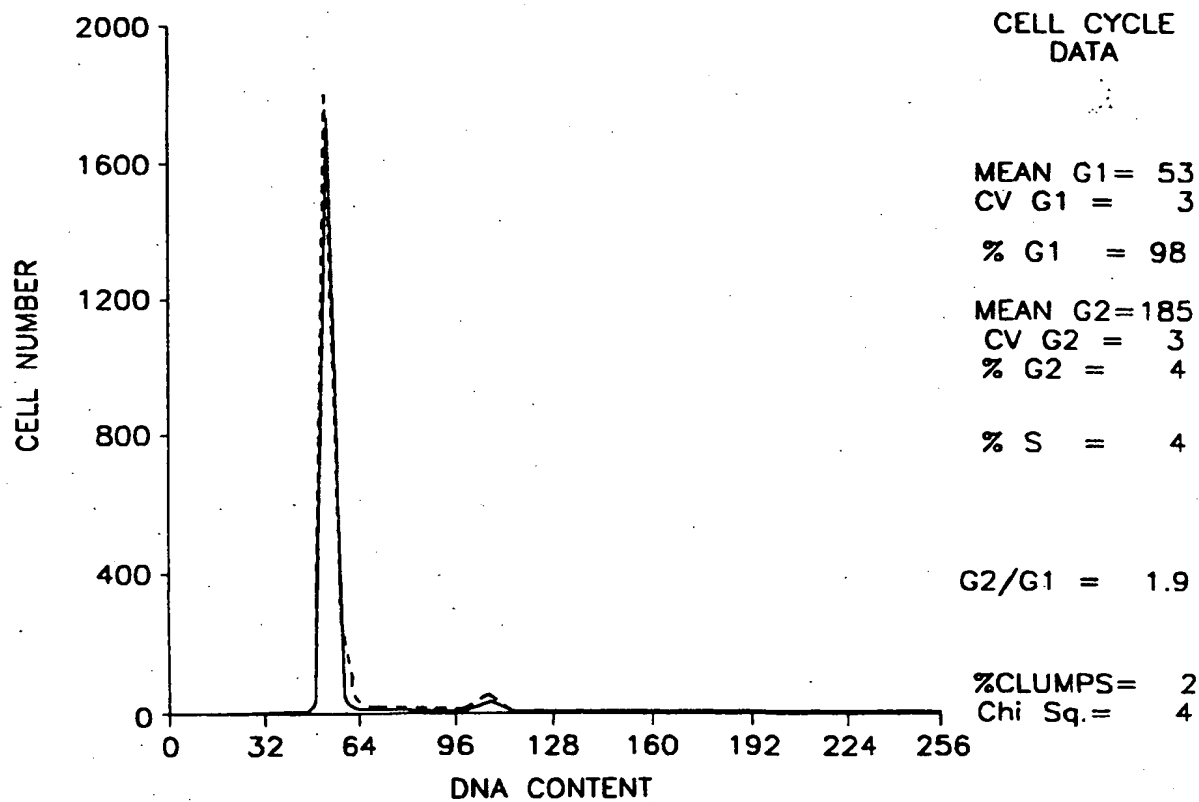


FIG. 23B

SUBSTITUTE SHEET (RULE 26)

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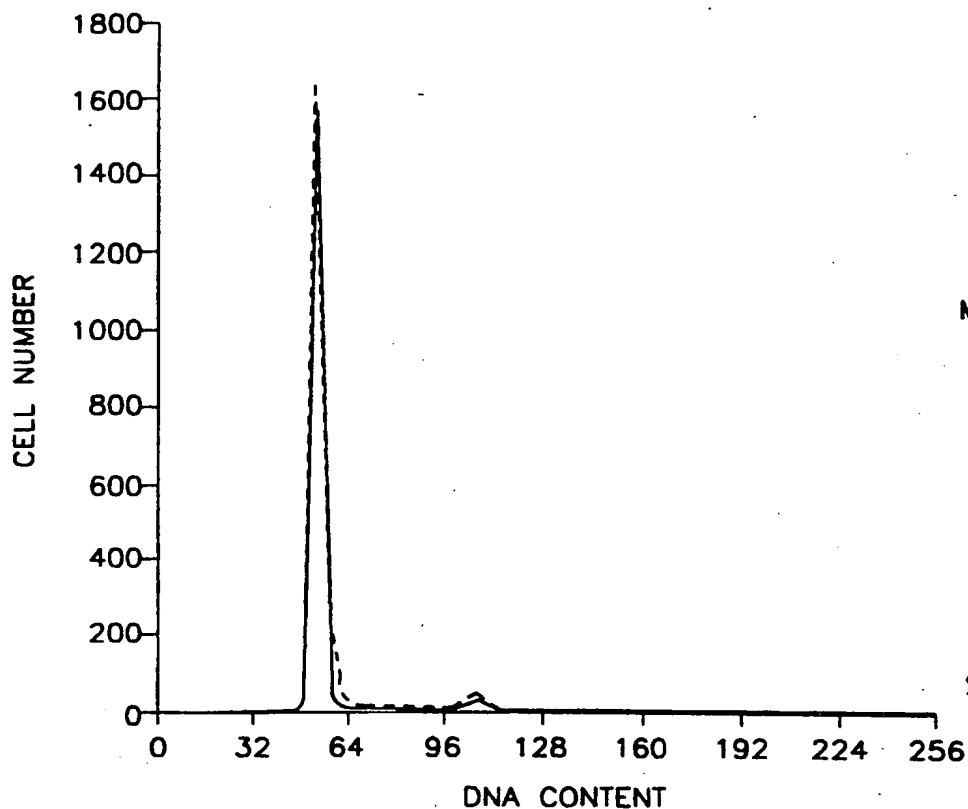


FIG. 23C

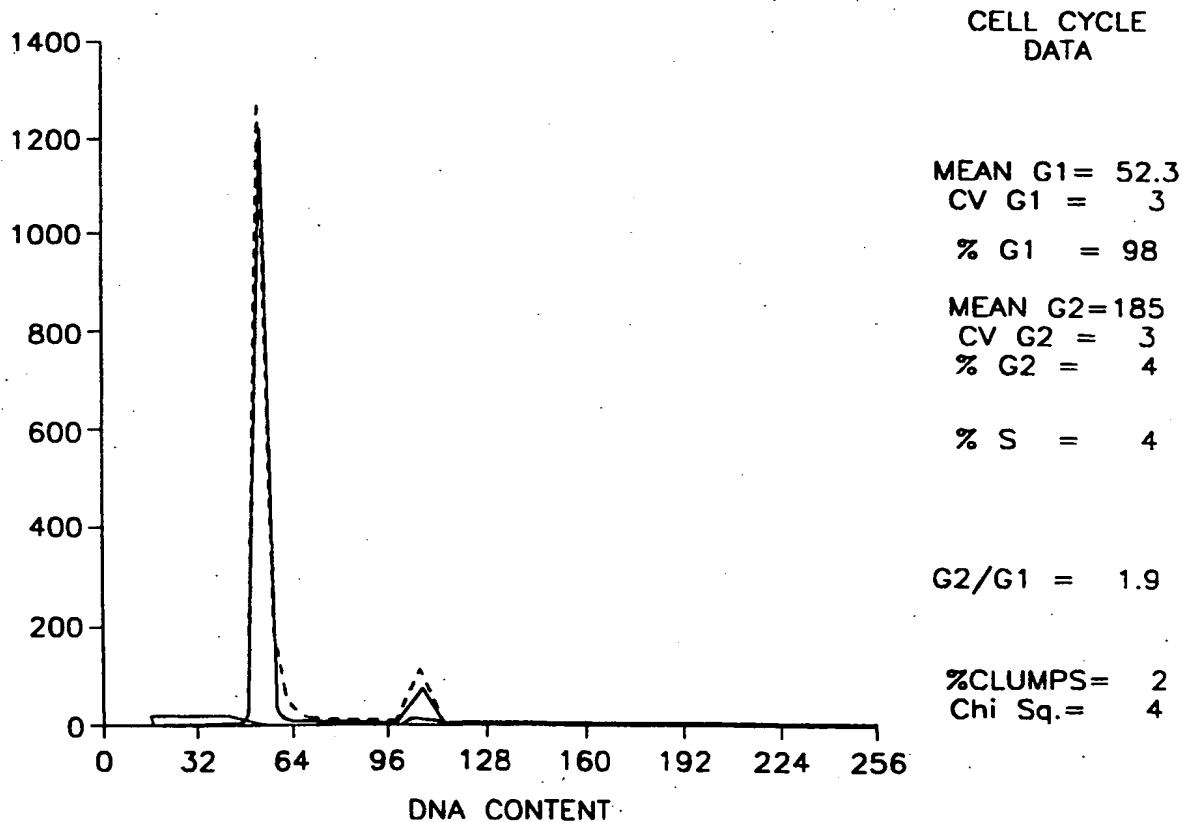
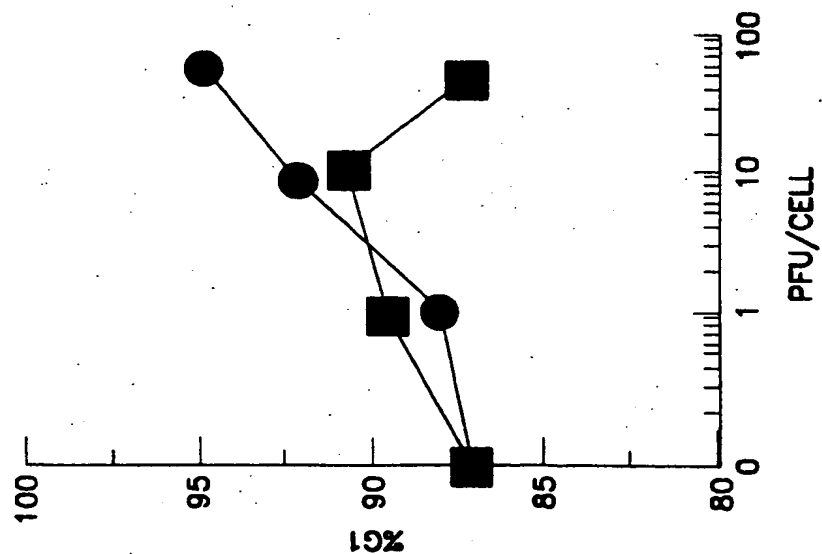
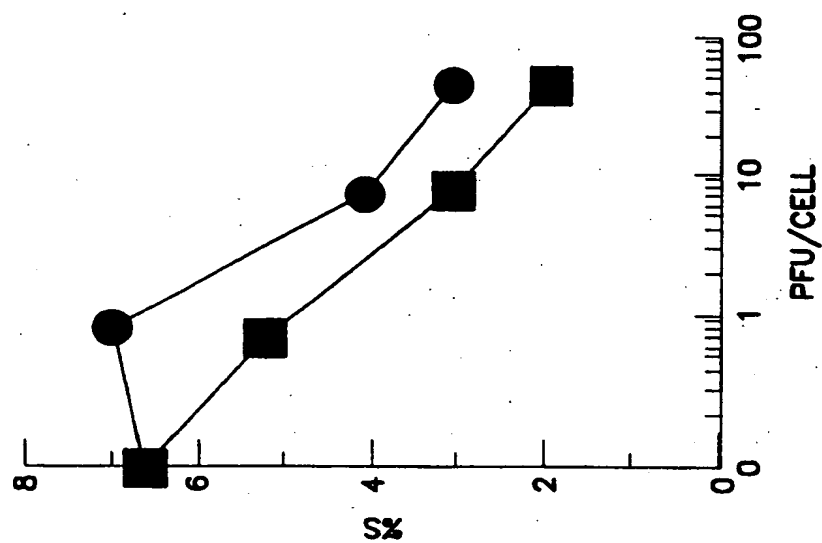
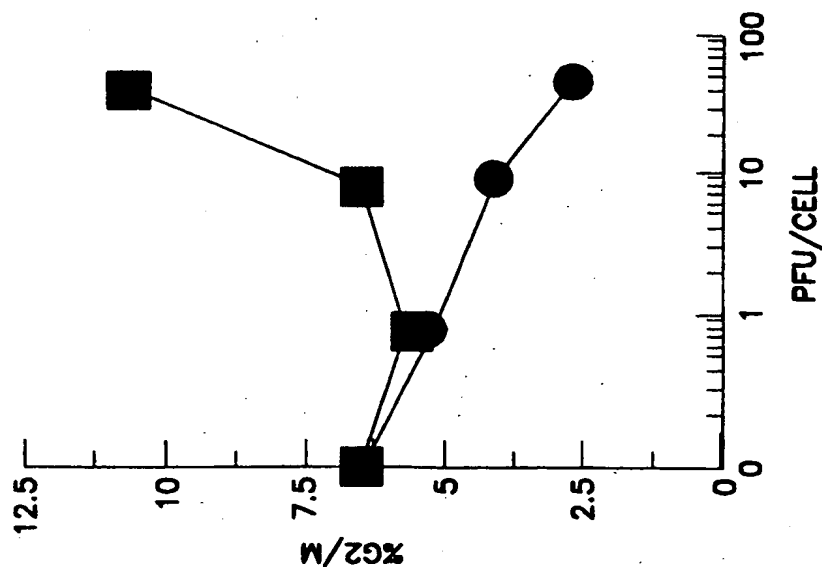


FIG. 23D

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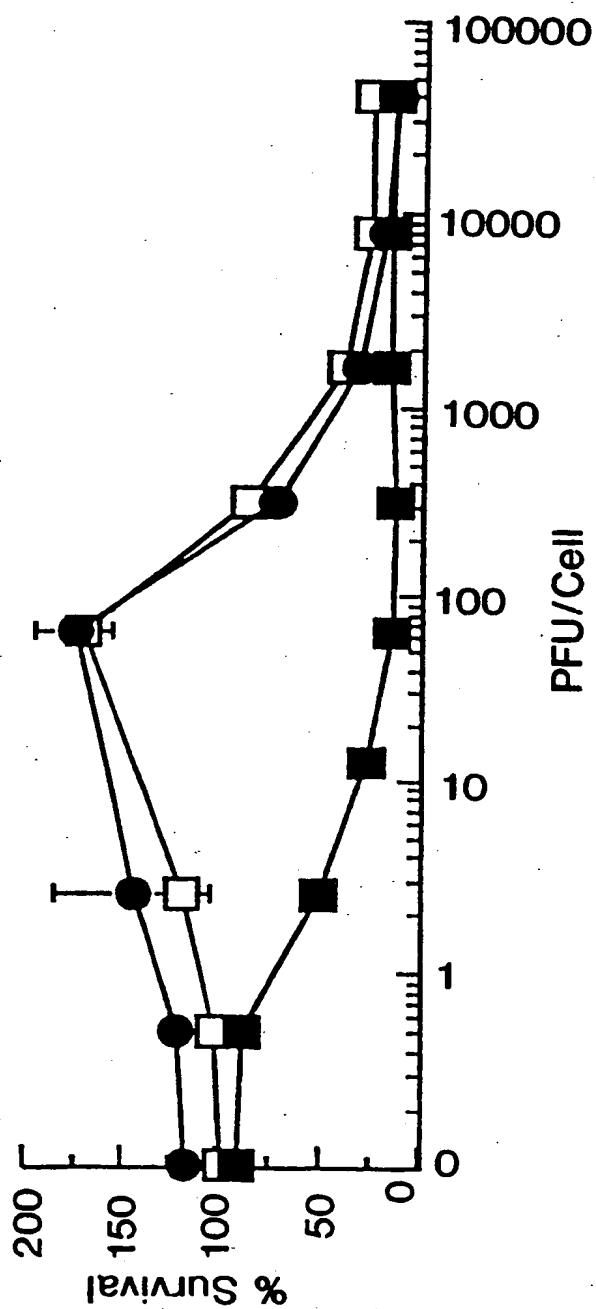


FIG. 24

SUBSTITUTE SHEET (RULE 26)

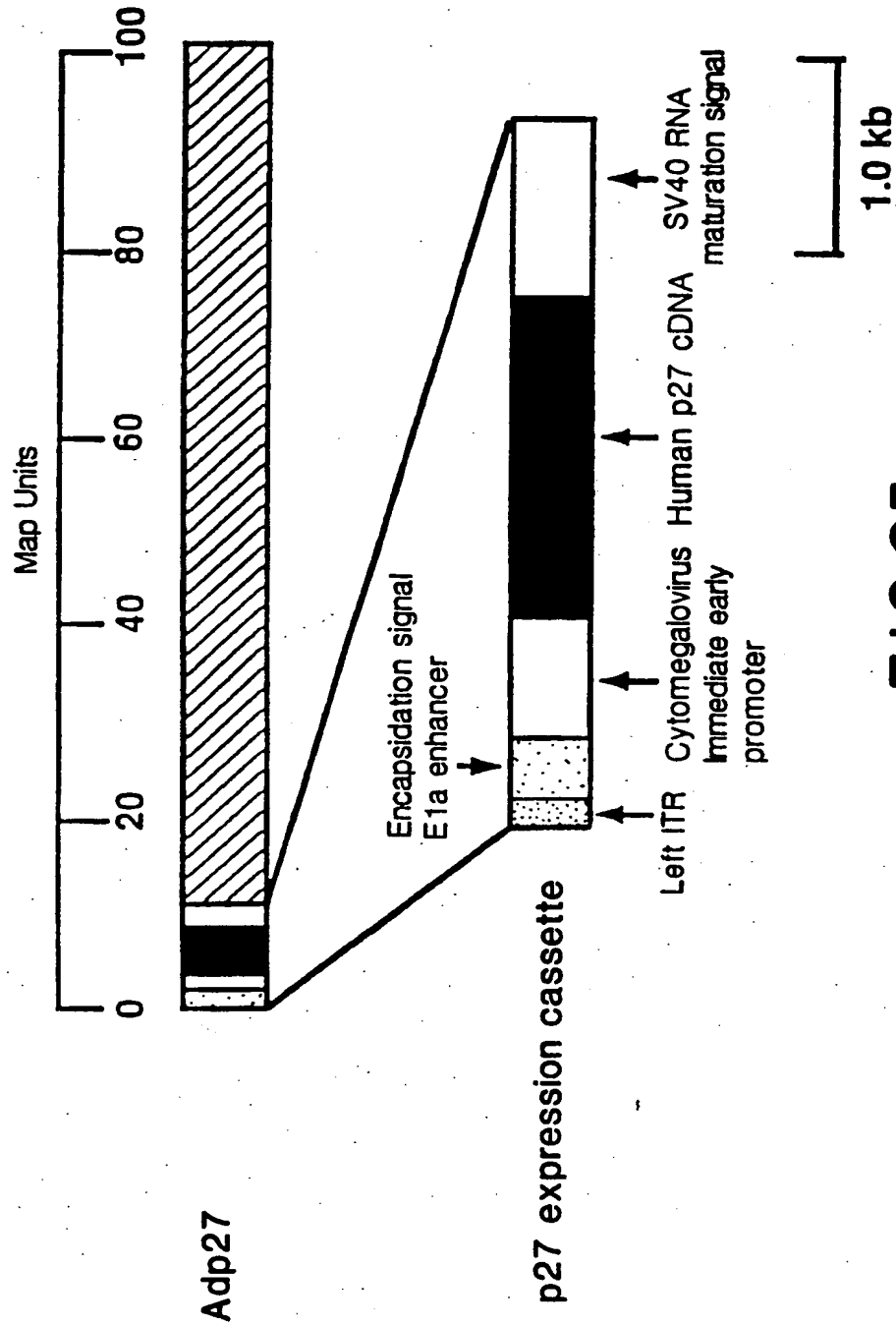


FIG.25

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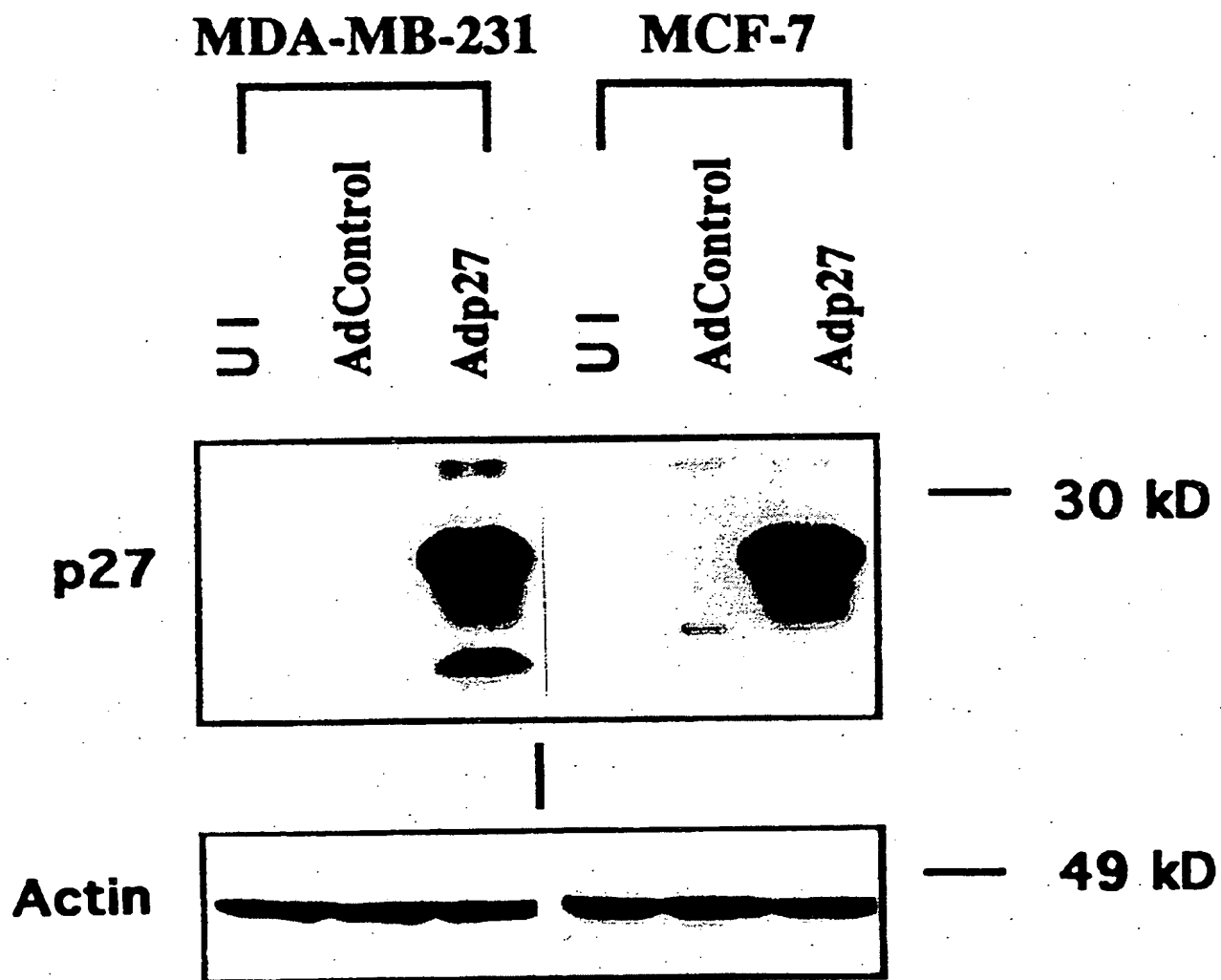


FIG. 26

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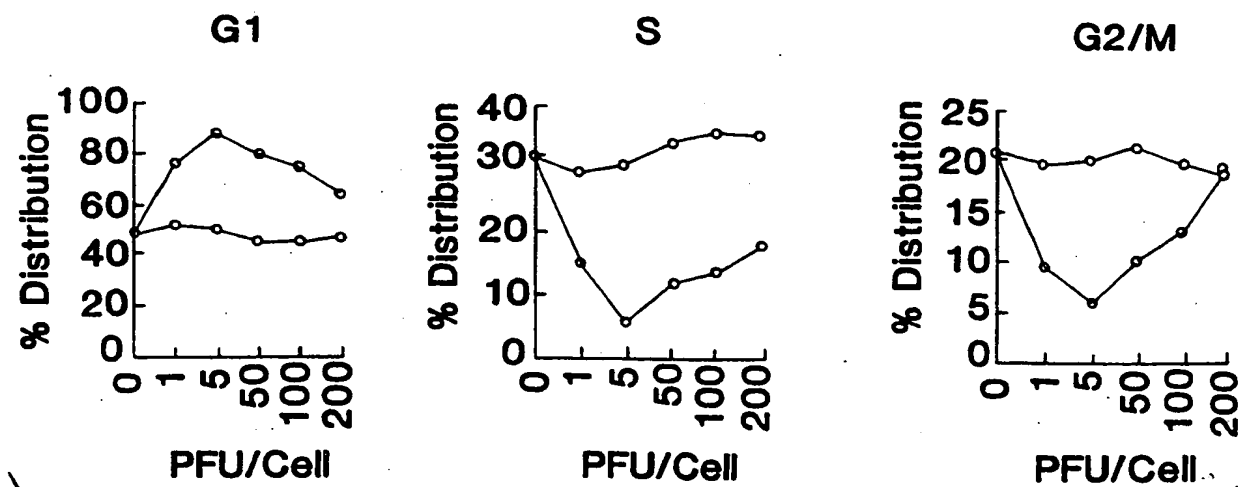


FIG. 27A

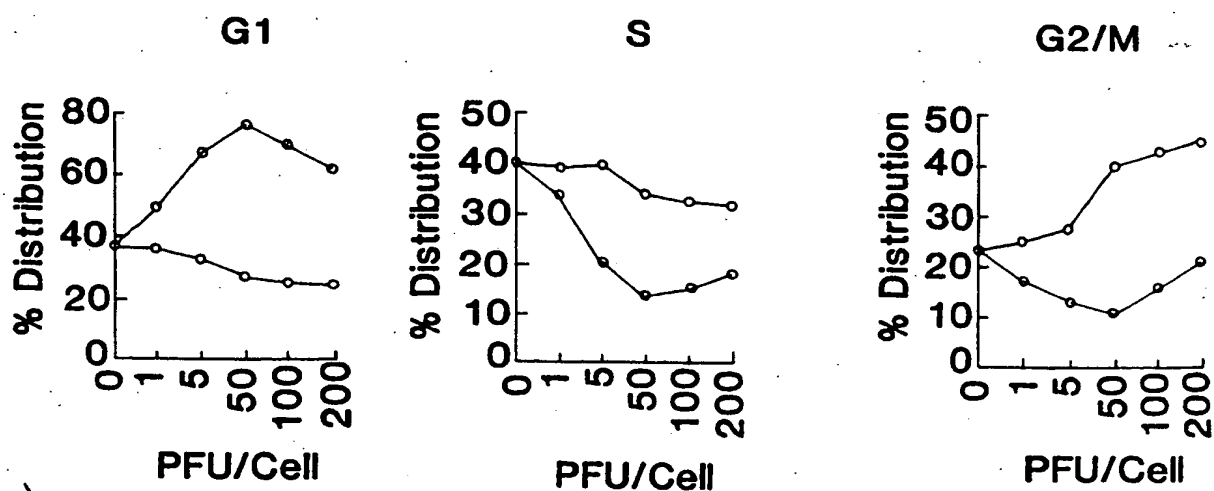


FIG. 27B

SUBSTITUTE SHEET (RULE 26)

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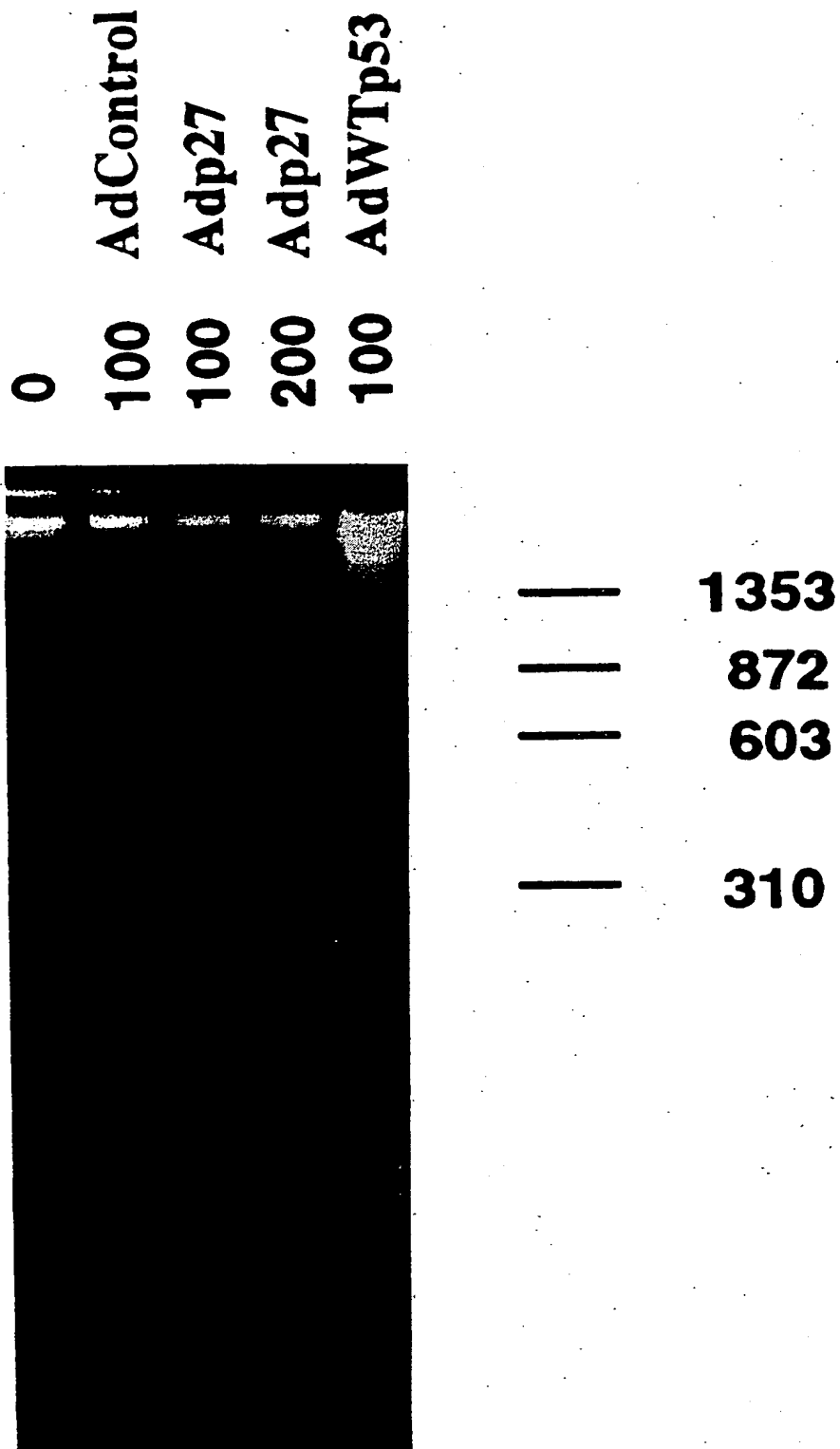


FIG. 28

SUBSTITUTE SHEET (RULE 26)

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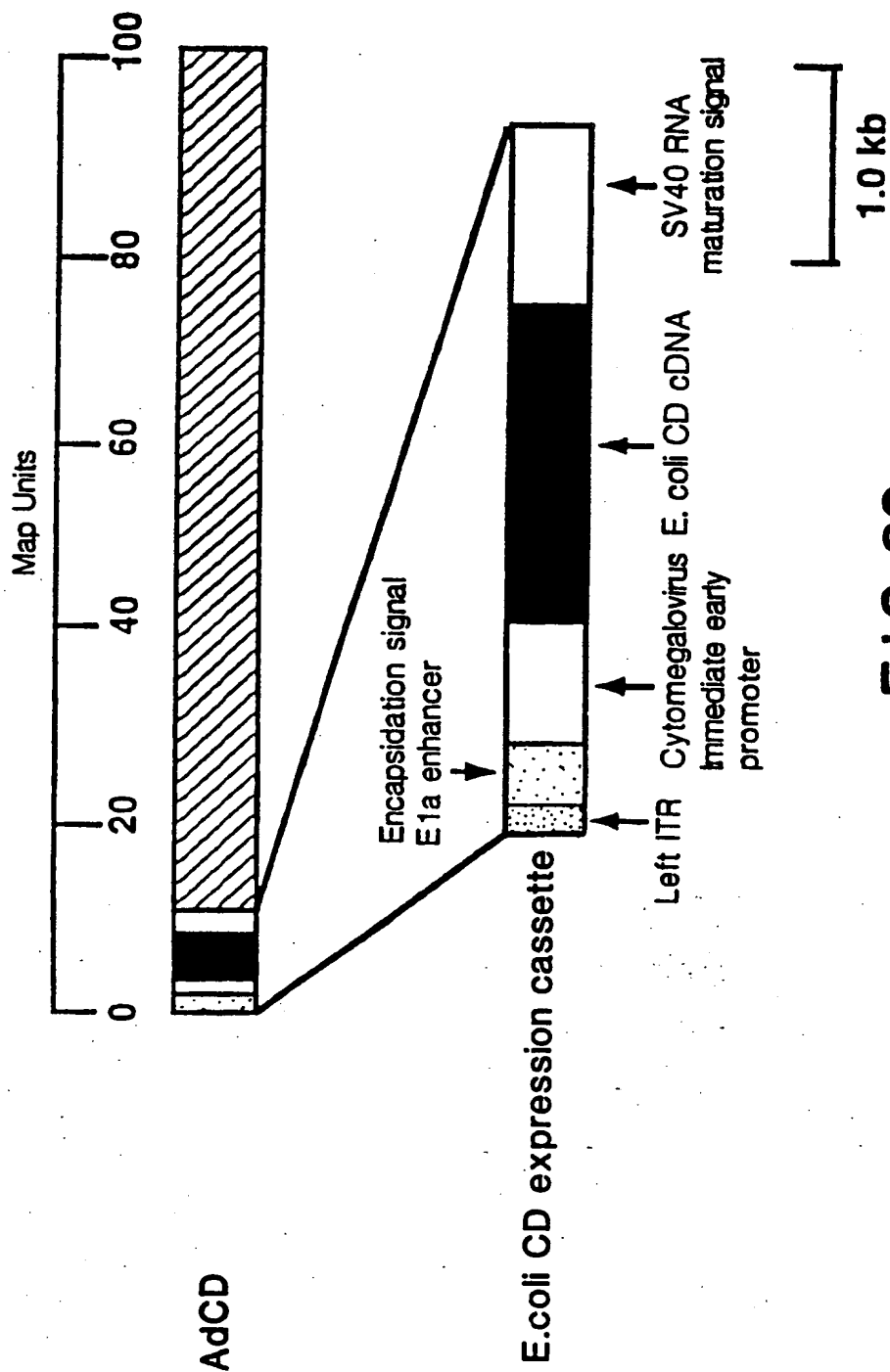


FIG. 29

SUBSTITUTE SHEET (RULE 26)

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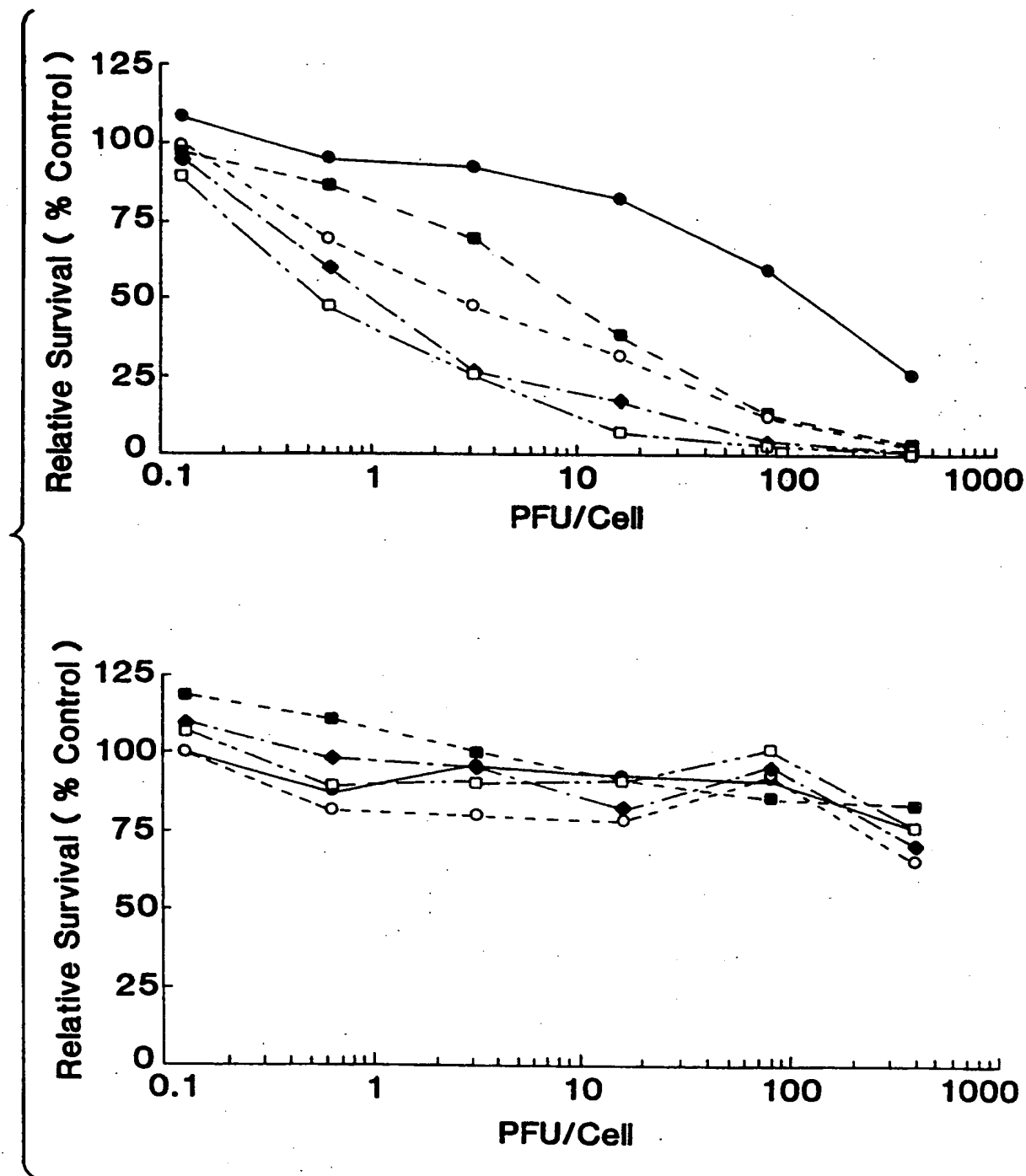


FIG. 30A

SUBSTITUTE SHEET (RULE 26)

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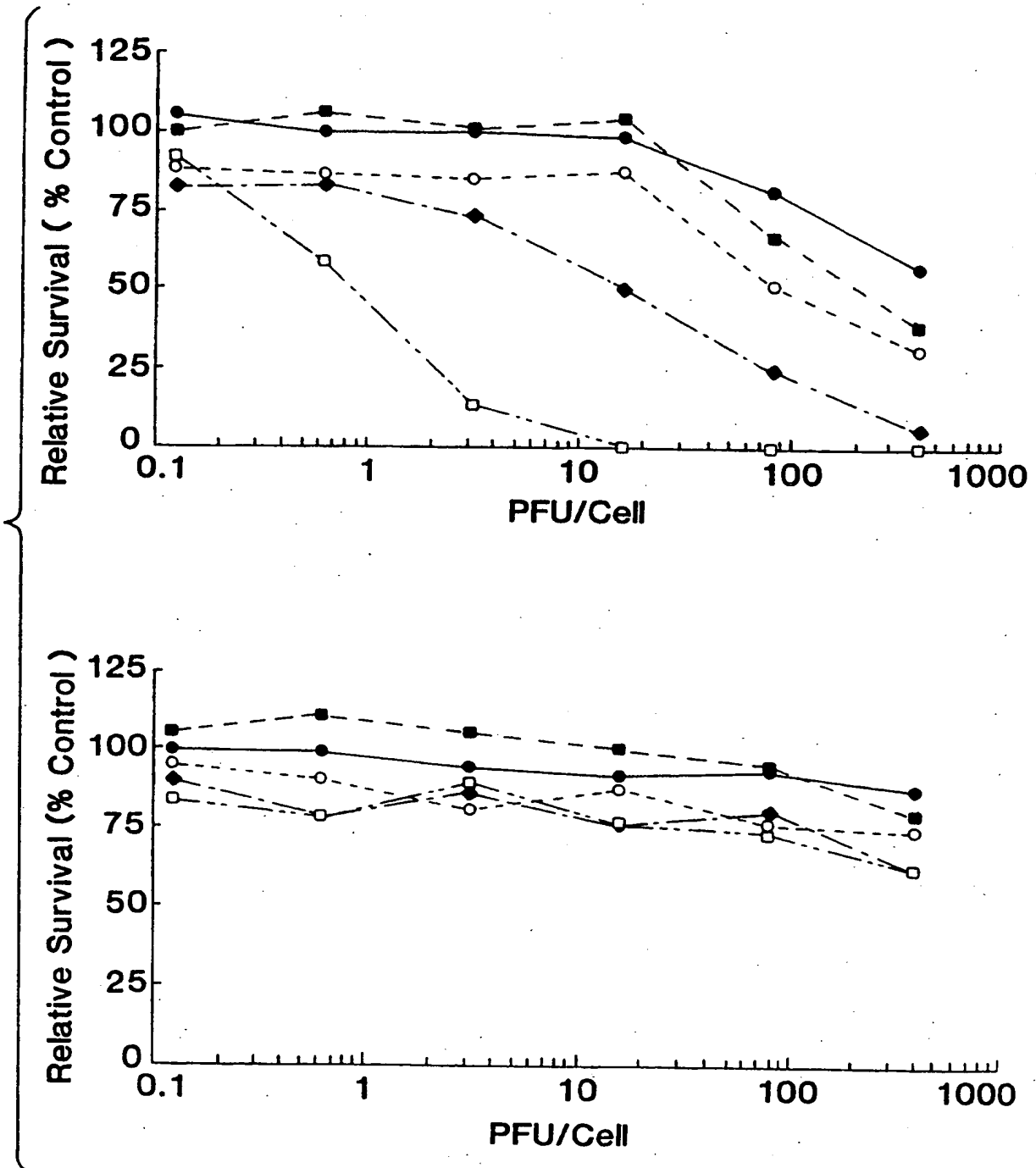


FIG. 30B

SUBSTITUTE SHEET (RULE 26)

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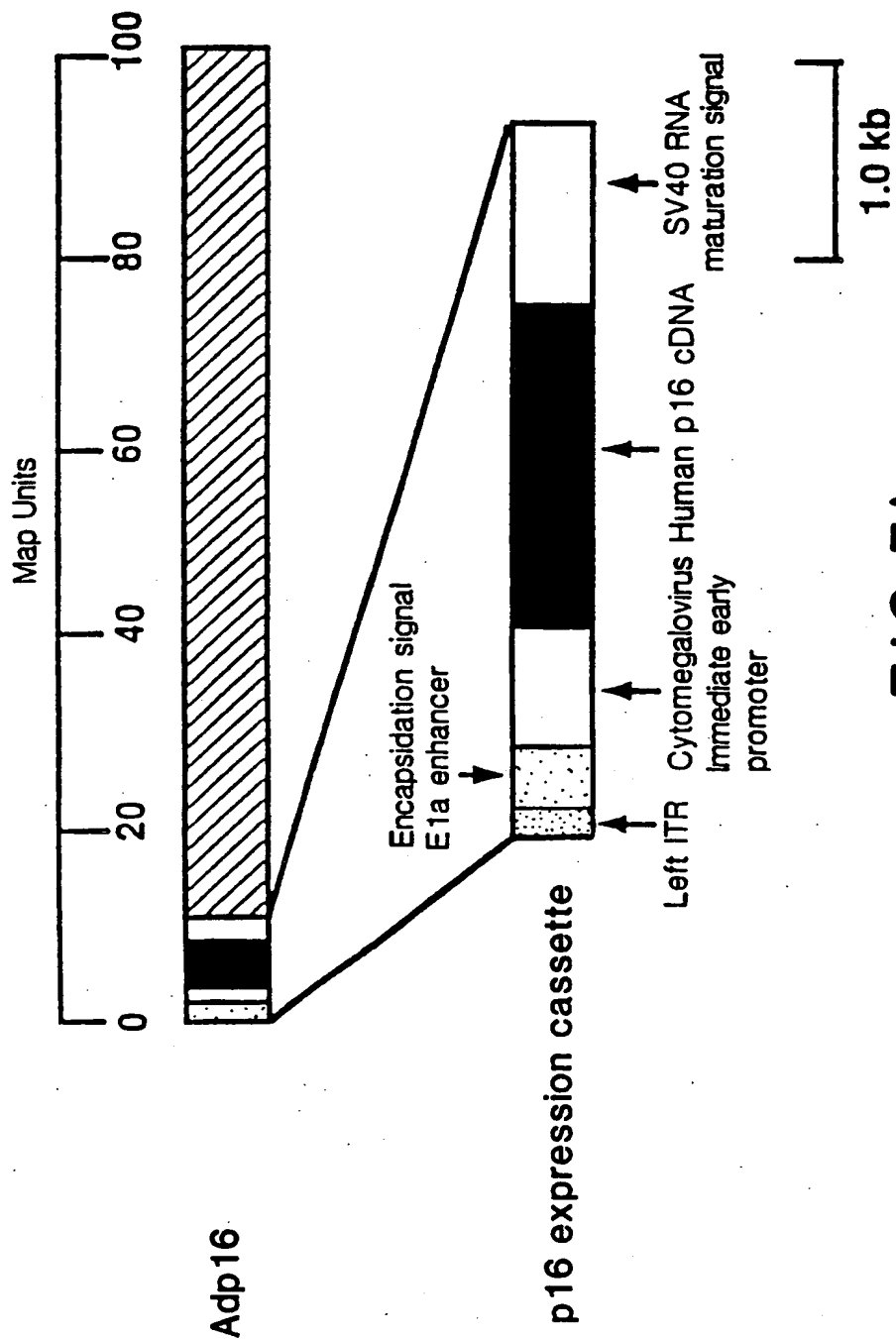


FIG. 3I

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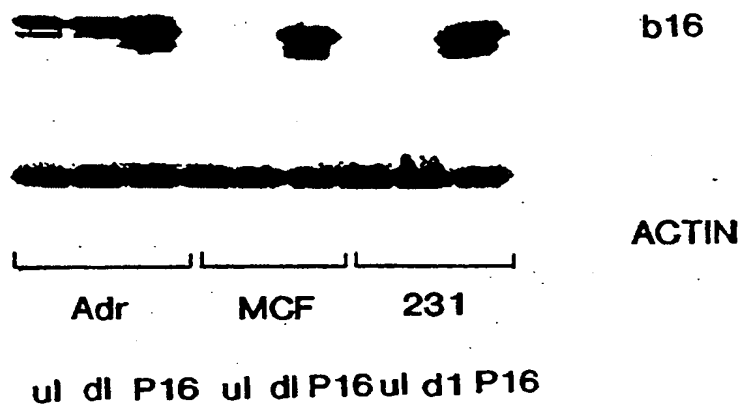


FIG. 32

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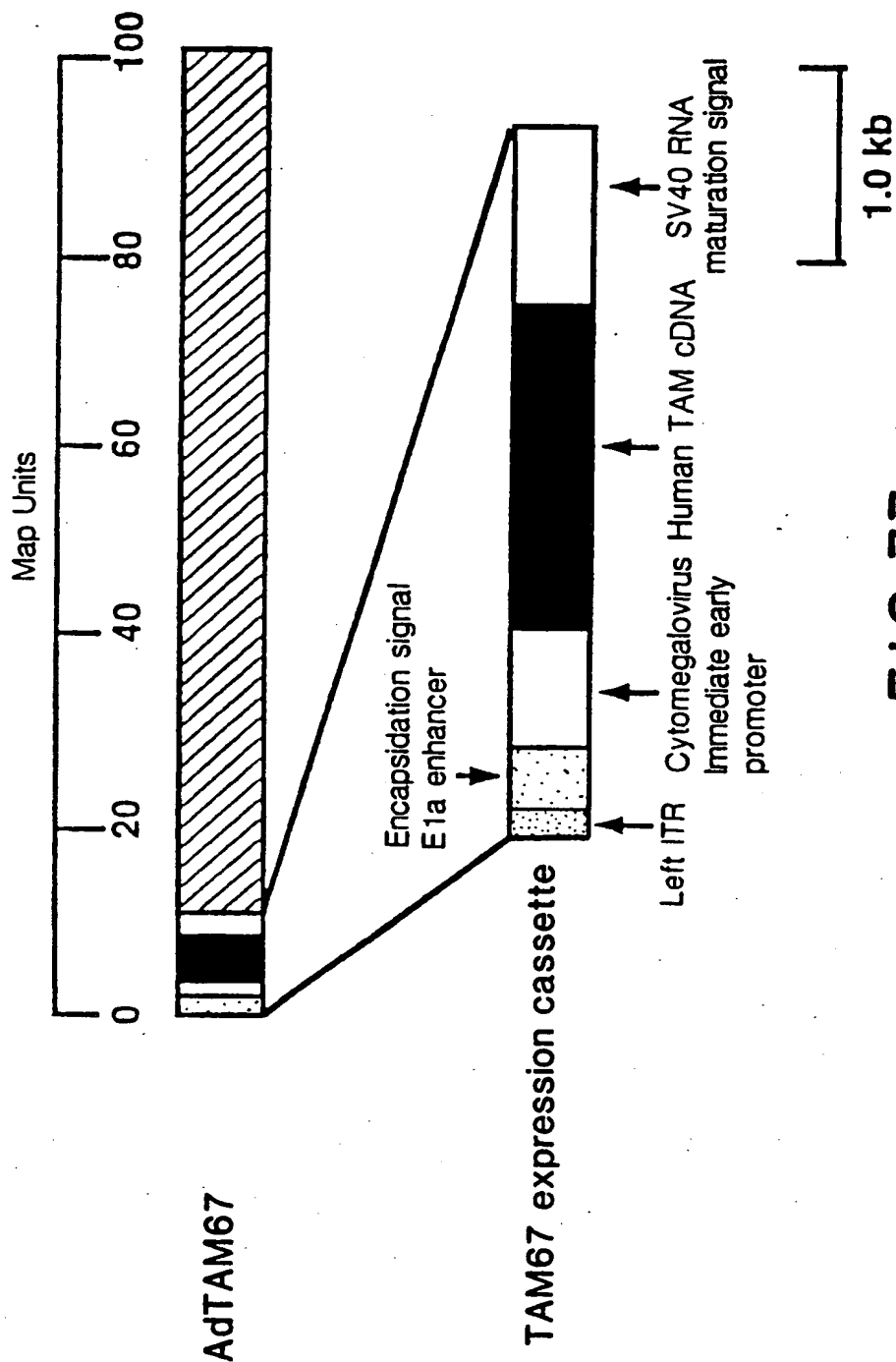


FIG. 33

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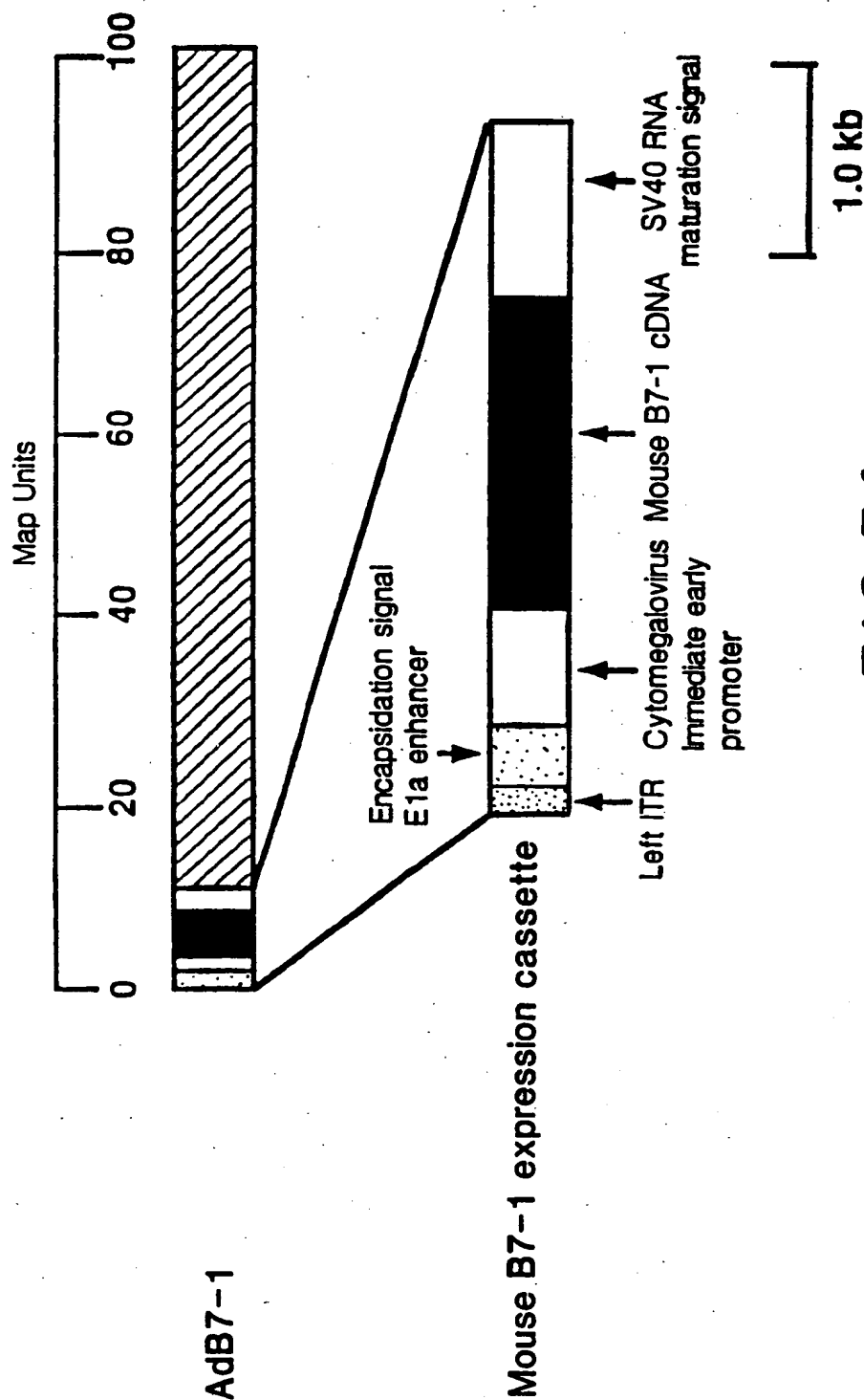


FIG. 34

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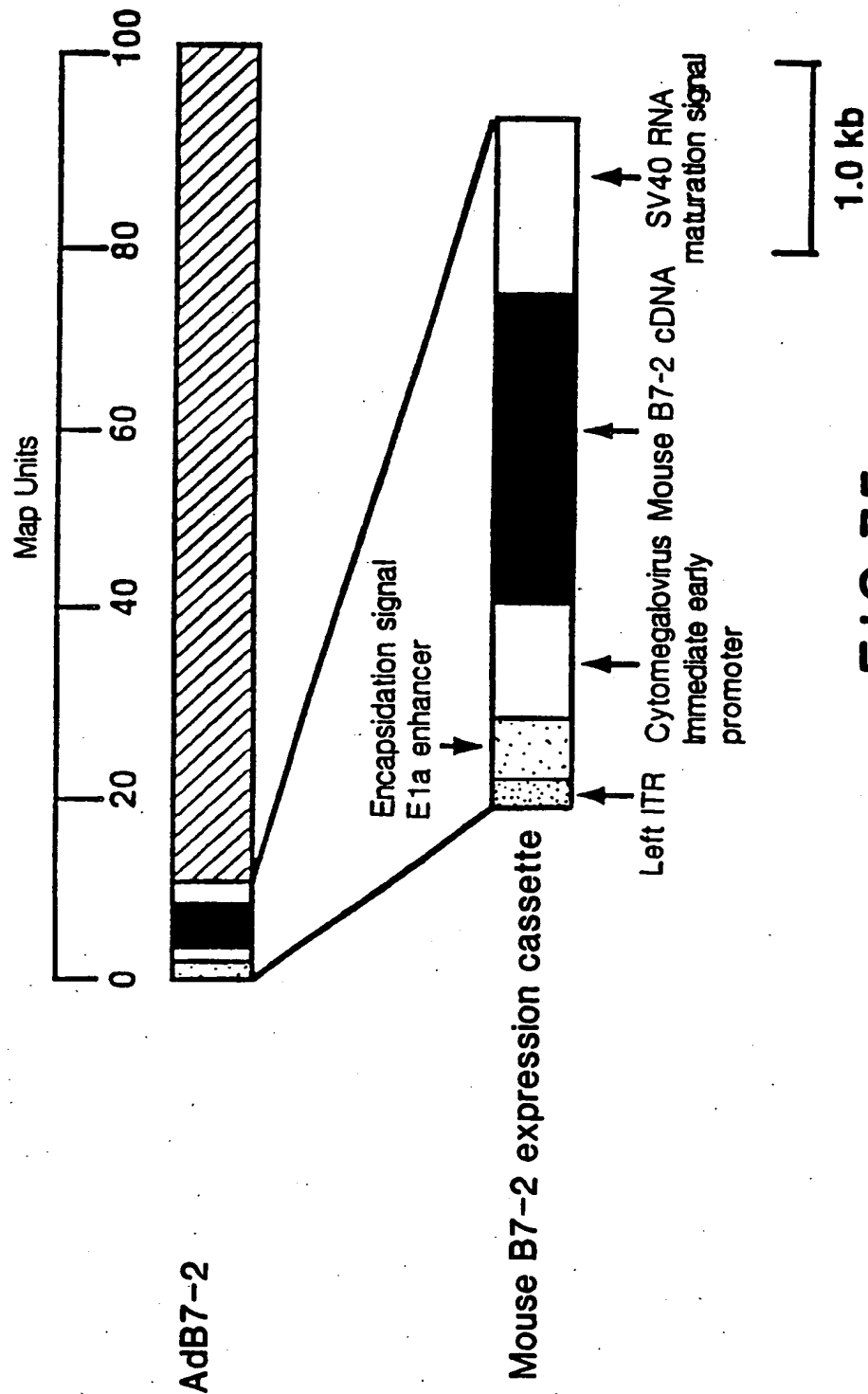


FIG. 35

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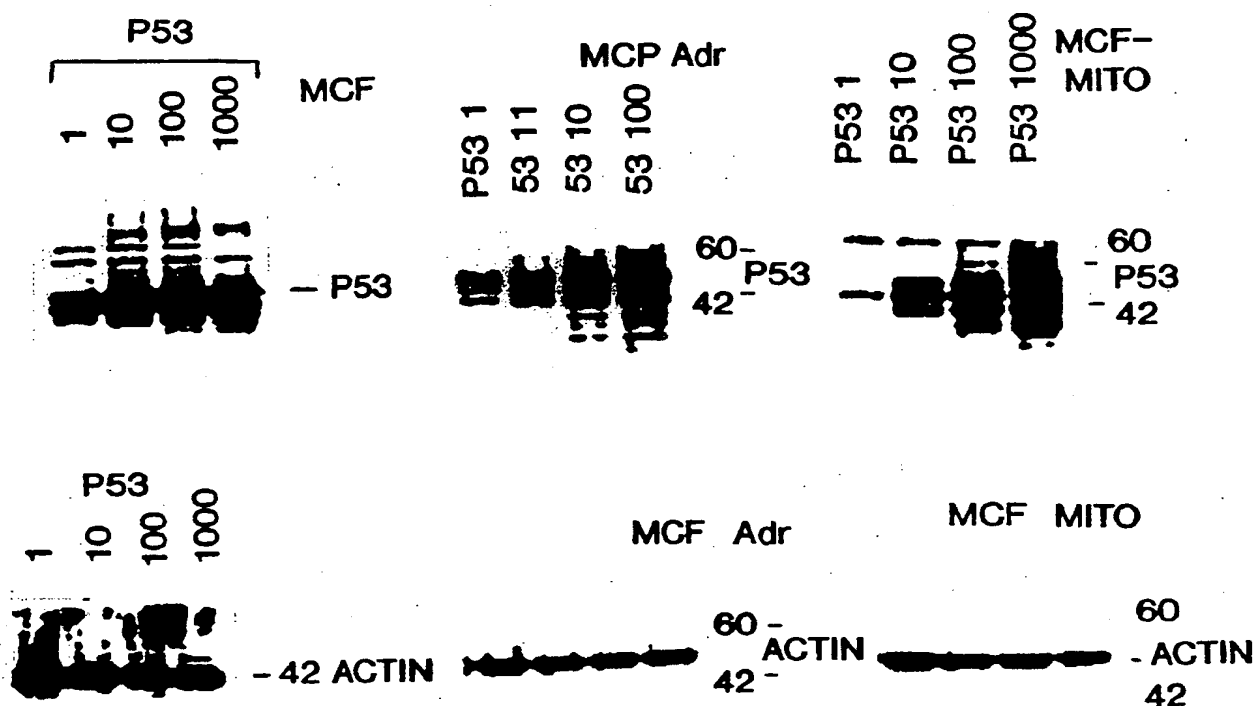


FIG. 36

SUBSTITUTE SHEET (RULE 26)

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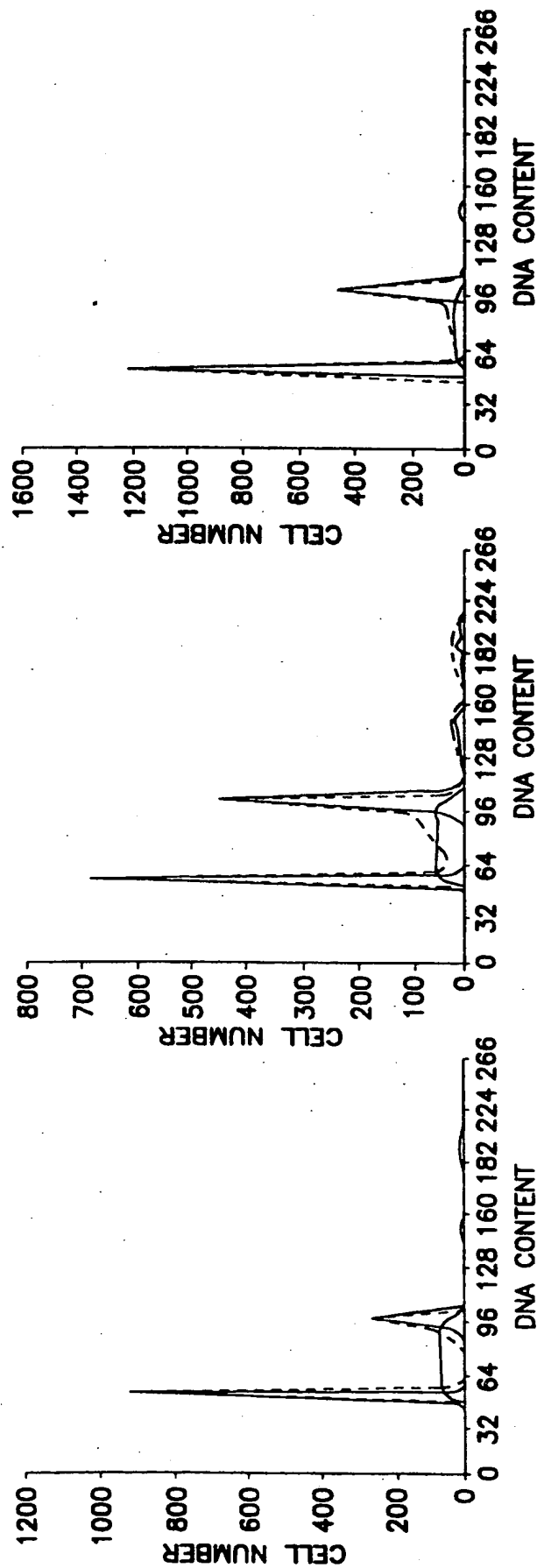


FIG. 37C

FIG. 37B

FIG. 37A

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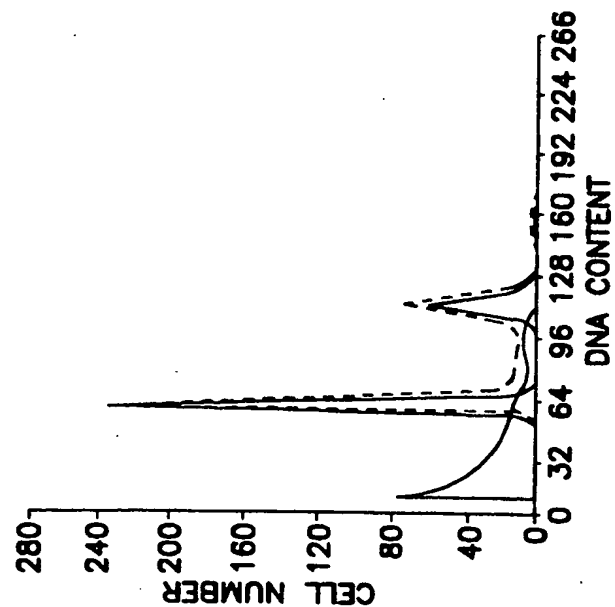


FIG. 37F

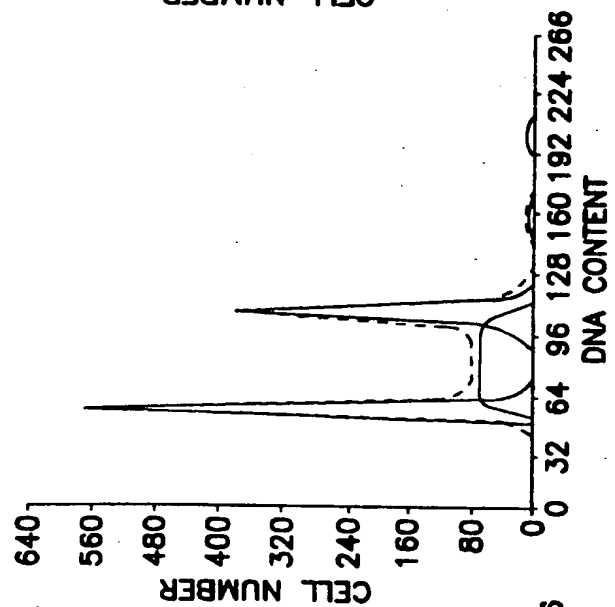


FIG. 37E

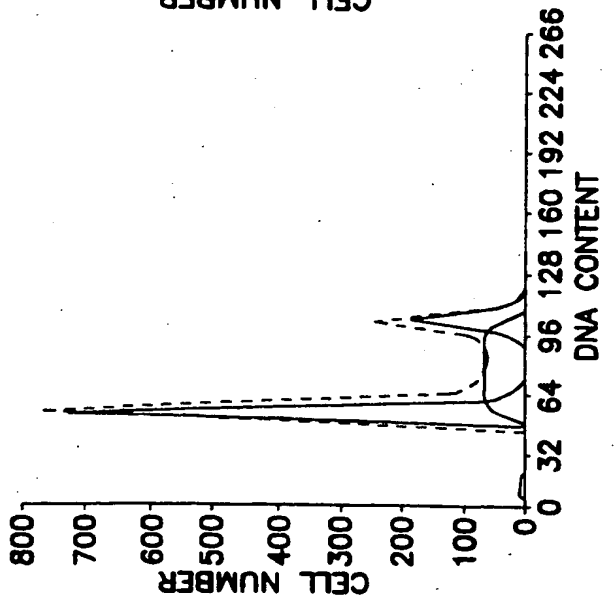


FIG. 37D

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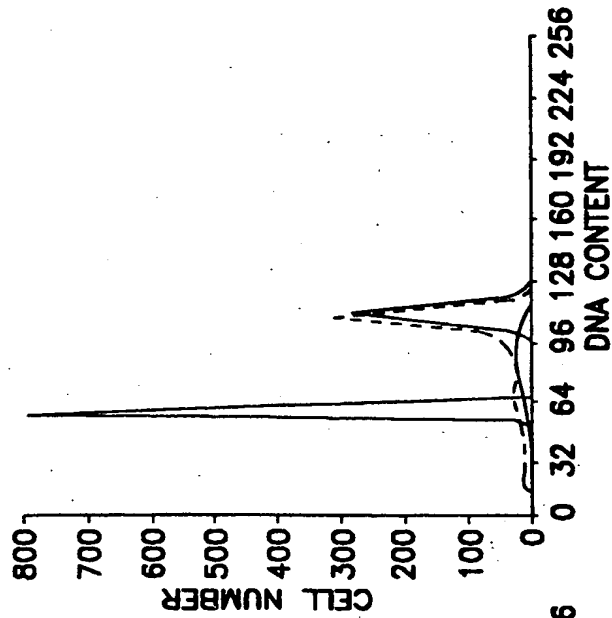


FIG. 37I

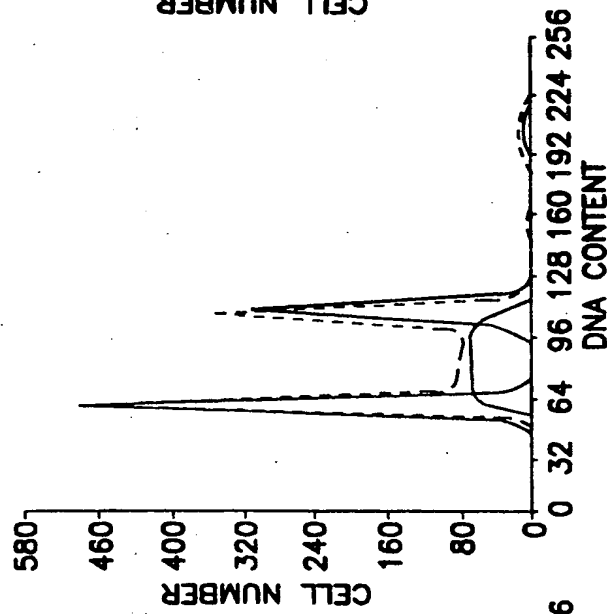


FIG. 37H

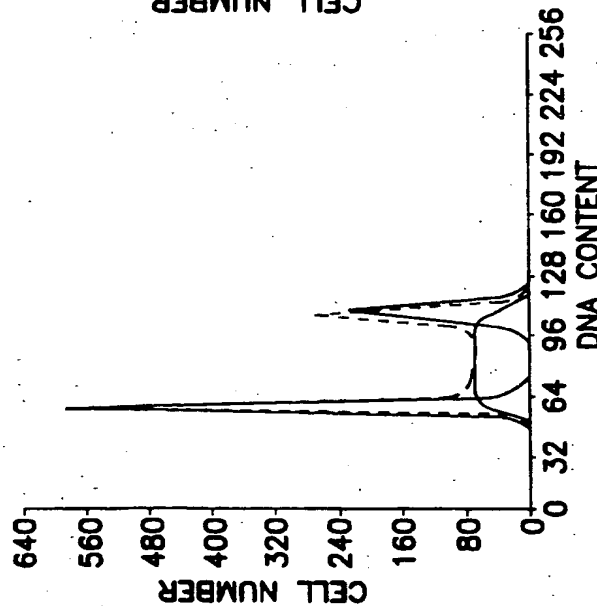


FIG. 37G

SUBSTITUTE SHEET (RULE 26)

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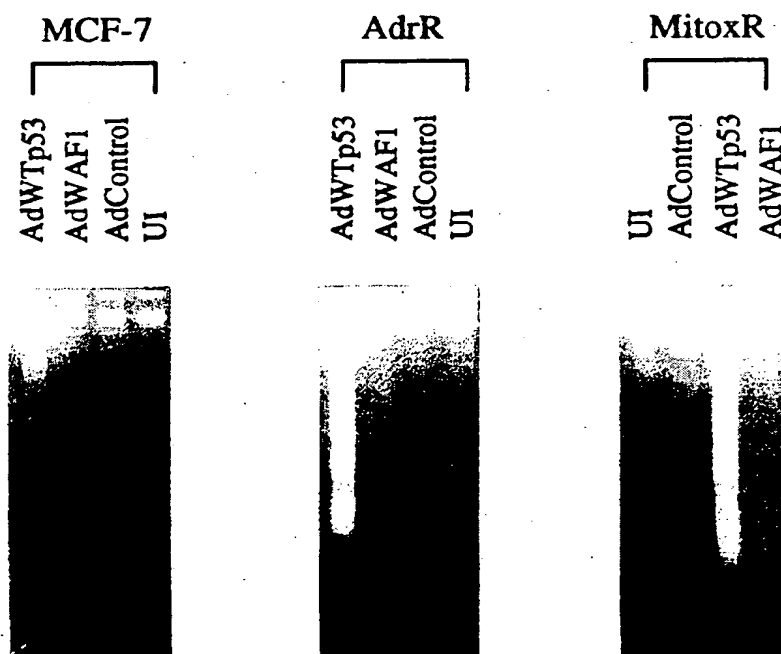
$$\begin{array}{r} 15^3 \\ 2 \\ \hline 306 \end{array}$$


FIG. 38

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No

PC 96/02336

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 A61K48/00 C07K14/47 C07K14/82 C12N15/12
 C12N9/78 C12N15/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRENDS IN BIOTECHNOLOGY, vol. 8, no. 4, 1990, pages 85-87, XP002012682 F. GRAHAM: "Adenoviruses as expression vectors and recombinant vaccines" see figure 2 ---	8-15
X	IN VIVO, vol. 8, 1994, pages 755-770, XP002012683 W. ZHANG ET AL.: "Anti-oncogene and tumour suppressor gene therapy - examples from a lung cancer model" see page 762 - page 765 --- -/--	8,10-15, 23-27, 29-46

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

12 September 1996

Date of mailing of the international search report

20.09.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+31-70) 340-3016

Authorized officer

Skelly, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/02336

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 24297 (PERRICAUDET) 27 October 1994 see the whole document ---	8,10-15, 23-27, 29-46
E	WO,A,96 15245 (ARCH DEVELOPMENT CORPORATION) 23 May 1996 see example 5 ---	8-15,23, 32
X,P	WO,A,95 13375 (THE JOHNS HOPKINS UNIVERSITY) 18 May 1995 see the whole document ---	8-13,15, 16,23-46
X,P	WO,A,95 10623 (GOVERNMENT OF THE UNITED STATES OF AMERICA) 20 April 1995 see page 9, line 6-13 ---	8,10-15, 23-27, 29-46
X,P	WO,A,95 18824 (SLOAN KETTERING INSTITUTE FOR CANCER RESEARCH) 13 July 1995 see page 34 - page 35 ---	9,10,15, 17,23-46
X,P	CANCER RESEARCH, vol. 55, 1995, pages 3250-3253, XP002012684 X. JIN ET AL: "Cell cycle arrest and inhibition of tumour cell proliferation by the p16ink4 gene mediated by an adenovirus vector" see the whole document ---	1-15,19, 23-46
X,P	BIOCHEM. BIOPHYS. RES. COMMUN., vol. 215, no. 2, 1995, pages 446-451, XP002012685 D. KAYATOSE ET AL.: "Consequences of p53 gene expression by adenovirus vector on cell cycle arrest and apoptosis in human aortic vascular smooth muscle cells" see the whole document ---	1-16, 23-46
X,P	CLINICAL CANCER RESEARCH, vol. 1, 1995, pages 889-897, XP002012686 D. KAYATOSE ET AL.: "Cytotoxic effects of adenovirus-mediated wild-type p53 protein expression in normal and tumor mammary epithelial cells" see the whole document ---	1-15, 24-28
X,P	WO,A,95 12660 (UNIVERSITY OF TEXAS) 11 May 1995 see the whole document -----	8,10-15, 23-27, 29-46

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/02336

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 23-30 (partially as far as they concern an in vivo method) 32-42, are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PC 96/02336

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9424297	27-10-94	FR-A- 2704234	28-10-94
		AU-A- 6572194	08-11-94
		CA-A- 2158869	27-10-94
		EP-A- 0695360	07-02-96
		FI-A- 954966	18-10-95
		HU-A- 73464	28-08-96
		NO-A- 954132	17-10-95
		ZA-A- 9402778	09-01-95
WO-A-9615245	23-05-96	AU-A- 4502096	06-06-96
WO-A-9513375	18-05-95	NONE	
WO-A-9510623	20-04-95	AU-A- 8017394	04-05-95
WO-A-9518824	13-07-95	AU-A- 1525195	01-08-95
		AU-A- 2770695	16-02-96
		WO-A- 9602140	01-02-96
WO-A-9512660	11-05-95	AU-A- 8094994	23-05-95
		CA-A- 2174556	11-05-95
		EP-A- 0725791	14-08-96
		NO-A- 961696	26-06-96